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Only self-help for British universities

The Committee of Vice-Chancellors and Principals is right to have lobbied the British government last week with its argument that a slower contraction of the university system would help not merely the universities but the government itself (see page 281). But nobody should be surprised if the government chooses not to respond. For ever since it was announced nearly a year ago that the allocation of public funds to the University Grants Committee would be reduced over three years by 8.5 per cent, it has been clear that the agenda of the dispute between the government and the universities is partly a hidden agenda. The government is behaving as if large parts of the university system are effete, and that the time has come to teach them a lesson. In public, however, the government merely says that higher education must share in the sacrifices expected, at times of economic decline, by other publicly supported institutions. It would be better for all concerned if the hidden agenda were now made public. The plight of the universities is worsened because the British government's secret opinion of their usefulness is broadly based. The universities have long since lost the wholehearted friendship of the major political parties. Student rebellions in the 1960s, although modest by international standards, are well remembered. The rash of new undergraduate courses that came in at the same time helped to undermine the reputation of the university system as a whole. That many academics and institutions stood out against these excesses is forgotten, unjustly but understandably. And even though some of the surviving industries in Britain which owe their chances of future prosperity to graduates from the universities are quick to acknowledge their dependence, the public impression of the utility of the system is more accurately shaped by the numbers of graduates in sociology and related fields who leave their graduation ceremonies to join the dole queues.

The issue of academic tenure, the substance of the vice-chancellors' complaint last week, is more contentious than the universities appear to understand. When the number of people unemployed in Britain is certain to increase above three million, it is hard to see emerging a wave of public sympathy for academics who lose their jobs. Indeed, the system of academic tenure is widely resented, even though it may be an essential assurance of freedom in scholarship — and, technically, required by the statutes under which many universities are established. Why should academics have a guarantee of lifelong employment when other people are being thrown out of work in droves? Against this public view, it would require a Hercules among ministers to persuade his colleagues in the British government to provide anything like a fund of £250 million to help academics see in the sunset years. Mr Mark Carlisle, Secretary of State for Education, is unlikely to fight for the vice-chancellors' case as they would wish. Such a fight would in any case expose him to the charge of maladministration — the muddle now emerging is, after all, one that he and his officials should have anticipated. Thus the most likely outcome of last week's meeting is that, for the time being, the government will let the universities stew in their own juice. If, in due course, the cost of academic redundancy threatens to loom large but the general level of unemployment has not declined, the government could think of overriding the provisions of tenure in academic contracts by means of legislation. As things are, the universities are virtually powerless. They have no choice but to seek out ways in which they can help themselves.

The first need is of self-help in the impending crisis. If the vice-chancellors' estimate of the number of academics likely to be

thrown out of work is anything like correct, the chances are that many people with a potentially important contribution still to make to scholarship and teaching will find themselves on a labour market in which their skills are not appreciated. Is it beyond the wit of the universities collectively at least to set up machinery for making sure that such displaced academics are not needed elsewhere in the system? And since all three parties to such an arrangement — the displaced academic and the two institutions between which he might be shuttled — stand to benefit, would not a fund to which all universities contribute help to smoothe the transfer of people between institutions? It is disappointing that the vice-chancellors seem so quickly to have abandoned hope that such schemes can function. It is also disappointing that they have not yet formally explored with the unions more radical departures from present arrangements — the replacement of annual by ten-month contracts, for example, or the feasibility of a negotiated reduction of salaries, at least in the institutions most directly threatened. The sheer rigidity of the present arrangements, under which academic (and other) university salaries are nationally uniform and tightly linked with age is in itself one of the causes of present troubles. In present circumstances, there is every chance that a majority of university teachers would prefer flexibility and even hardship to the collapse of the institutions to which they belong. That chance should be explored, and quickly.

Another immediate need is that universities should work out ways of sharing resources with each other. The University Grants Committee has pointed to some of the more obvious possibilities — the pooling of equipment or the merging of departments between universities that happen to be close to one another. Other benefits could flow from joint appointments, the designation of some departments in some universities as places whose research equipment is regarded as a freely accessible pool. More important, in the contracting system there should be ways of pooling students, graduate students in particular. Some of the universities that survive the years ahead will be more lopsided than they should be. Some areas of study will have disappeared. Some departments, even whole universities, may find it necessary to give up in-house research. So the British university system, traditionally too indifferent to the need that students should have some freedom to explore different fields of study, needs to find ways of helping students as well as teachers move. And because it would be intolerable that students' chances of becoming graduate students should be determined by the chance that the university to which they happen to belong can keep its graduate programmes in being, there is an urgent need of national arrangements for placing bright people. This is a problem for the research councils.

It is not, however, too soon to ask what plans should be laid against the period after 1984–85, when last month's cuts have taken their full effect. For the causes of the present crisis will still then persist. Lacking coherence as a system and the capacity internally to be decisive even on academic policy, the university system of the late 1980s will be just as vulnerable as it has proved to be in the past few weeks. The most urgent need is that the system of public and private support for university education should be arranged in such a way that individual universities have a more explicit incentive to provide a valued service for their students and the communities in which they are embedded. Universities that are popular with students for good academic reasons should be helped financially — and not penalized (as the University Grants Committee is now threatening) if they attract more than their quota of home students. Good research, even as



crudely measured by the volume of successful research grant applications, should qualify for a bonus.

Breaking with tradition, the University Grants Committee should also make explicit the basis on which its allocations of funds are made, so that universities know how most successfully to compete for what funds there are. And something should be done to clarify, simplify and ideally cheapen the present cost to public funds of supporting students in universities. One of the causes of the government's discontent, and the immediate cause of the restriction on student numbers now announced, is the cost of paying student fees and maintenance grants, using local authorities as paying agents. These arrangements, dating back to the days when students were considered to be the salt of the earth, are certainly not the most economical ways of helping to encourage the spread of university education. The anomalies which they include are often inequitable as between one student and another. One of their consequences is that British universities are chronically short of student accommodation. The arrangements should be re-examined, whatever noise the National Union of Students makes.

But why should the universities take the initiative in helping the government to save money, when the government has been so beastly? That is a hollow argument. The objective is not economy but independence. For all its faults, the British university system is a remarkable and valuable institution. The most immediate danger is that it will be so demoralized by the three painful years ahead that its capacity for self-renewal will be permanently impaired, as will be its capacity to persuade those who must support it that even its present role, even in present circumstances, is indispensable. The most cruel irony is that the British university system has been so mindlessly attacked when it is more than ever apparent that Britain lacks many of the skills that the universities could cultivate.

Clinch River runs again

During President Carter's tenure at the White House, the fast reactor site at Clinch River in Tennessee seemed destined to become a special kind of white elephant — a collection of unused equipment surrounding a half-begun concrete structure that would for all time be a monument to the fancied folly of the 1960s that it would be possible economically to win power from a nuclear reactor making more fuel than it consumed. Now, the prospects are changing. Against expectations and the recent voting record of the key committees in the House of Representatives, Congress appears to be ready to see work at the reactor site begin again (see *Nature*, 16 July). President Reagan, having spoken fondly of Clinch River during his election, dutifully included in his budget in March a request to Congress for funds to start building again at Clinch River. But the issue has never seemed important enough to the Administration to justify the risks of a serious fight. In the end, local interests in Congress have given the Administration what it was asking for without blood being spilled. The embarrassment, for President Reagan, is that he must now ask the Department of Energy to brush up its policies in other parts of the domestic nuclear energy programme.

Although the completion of Clinch River has for some time seemed to be an acid test of the willingness of the United States to commit itself to fast reactor technology, appearances are false. The Clinch River design is more than a decade old; much has since been learned, especially in France, about the design and operation of fast reactors. The escalation of the cost of the project, by a factor of three or thereabouts, is a measure not so much of the optimism of its original designers as of the need for changes of design after construction had begun. The enforced four-year delay will not have helped. Clinch River may yet be a white elephant of a different kind, a representation of an earlier technology. Even so, it will provide some practical experience of the operation of nuclear reactors cooled by liquid metal (sodium). And it will be another spur to the continuing wrangle in the United States about the rights and wrongs of nuclear power.

Technically, Clinch River is a demonstration plant, intended to demonstrate to utilities and their customers that electricity can be produced safely from fast reactors and that, in due course, fast reactors could be built economically. The demonstration would have been more helpful if the design had been less out of date. But in the assessment the utilities will eventually have to make, one crucial piece of information will be missing. The economic case for breeder reactors of any kind (which need not be fast reactors) is that they can use plutonium as fuel. When President Carter took fright at the prospect of the "plutonium economy", the extraction of plutonium from spent fuel rods taken from existing reactors was suspended. The Administration's new and sensible policy on the proliferation of nuclear weapons (see page 281) will help to liberate the Department of Energy from the traditions of the recent past, but there is a lot to be done before the economics of the plutonium fuel cycle can be tested. Completing Clinch River will, however, be a nonsense if that is not quickly done.

The wider implications of Clinch River in the nuclear debate are not at this stage easy to foresee. The most serious argument in recent years against breeder reactors has been economic; the price of uranium has risen so much less quickly than that of fossil fuels, and the cost of electricity is so much less dependent on the cost of fuel in nuclear reactors than in fossil power plants, that the potential advantages of breeder reactors (fuel at no cost at all) are not worth striving for. So why not put the huge cost of development into more imaginative exploits? The argument is respectable, and deserves attention. Its flaw is its assumption that the relative prices of uranium and fossil fuel will remain what they are at present for two or three decades ahead. The snag, of course, is that the present price of uranium (about \$50 for a pound of uranium oxide) reflects the decline in the rate at which utilities have been ordering conventional nuclear plants in the past few years. If, as memories of Three Mile Island fade, and the lessons of that accident are properly learned, the utilities turn again to nuclear power as a source of electricity, the price of uranium would shoot up. Is it not, in such circumstances, prudent that there should be data about the operation of some breeder reactor that would allow sensible decisions to be made?

Less cogent arguments have been made by those who say that fast reactors are inherently less safe even than conventional nuclear plants. Anxiety of this kind stems from the observation that breeder reactors contain substantial amounts of fissile material, and that the neutrons involved in the nuclear chain reaction are fast and therefore, on the face of things, likely to complicate the control of fluctuations of reactor output. Neither argument is as persuasive as it seems, however. Weapons designers are always saying that their most serious problem is that of arranging that fissile material can be coaxed into some configuration that will explode, so that the risk that such a configuration will turn up by accident in a breeder core is likely to be small; certainly the risky configurations can with ingenuity be identified in advance; and steps can be taken to avoid them or to prevent the damage they would do. And anxiety about neutron speed should be offset by the relatively low thermal inertia of fuel elements in fast reactors and the high thermal capacity of the molten sodium. None of this suggests that there are no risks in completing Clinch River but merely that theoretical safety studies have probably now gone as far as they can.

The third foundation of the case against Clinch River, of which much will be heard in the weeks ahead, is that all nuclear reactors are an abomination and that fast breeders are a particular abomination because one of their products is plutonium, sometimes described as the "most poisonous substance on earth" and sometimes feared because it might be used for making weapons clandestinely. The arguments presuppose that neither the safety of nuclear plants nor the security of the material they produce can be held within reasonable limits. Yet even the colourful experience of Three Mile Island, essentially a demonstration that it is possible so seriously to damage a nuclear plant that it is virtually useless without scattering dangerous doses of radioactivity in the neighbourhood, cannot be used unambiguously to support the case against Clinch River and what might follow.

US revamps nuclear proliferation policy

Reagan's new plan: reactors for friends

Washington

President Reagan announced last week that he intends to shift the balance of US strategy back to a political rather than a technical approach for restricting the proliferation of nuclear weapons.

In contrast to President Carter, who sought non-proliferation objectives by threatening to withhold access to US nuclear materials and technology, Mr Reagan intends to emphasize "working to improve regional and global stability" and to use the influence which would be generated by the United States' role as a "predictable and reliable partner" in nuclear development.

He proposes to distinguish between different countries in the way in which non-proliferation policies are applied, a move which will please European and Japanese allies but could create problems elsewhere. Mr Reagan also said that his Administration "would not inhibit or set back civil reprocessing and breeder reactor development abroad"; but he added that this condition applied to "nations with advanced nuclear power programs where it does not constitute a proliferation risk".

This was a more moderate statement than some which the President made during his election campaign. The shift seems to have been due not only to a greater awareness of the complexity of non-proliferation, but also to the implications of the Israeli raid on the Iraqi research reactor, which Mr Reagan referred to obliquely in his statement when he talked of the urgency being "highlighted by the ominous events in the Middle East".

But the statement still gives critics of the Administration plenty to chew on. For example, the President announced that he intended strongly to support the efforts of the International Atomic Energy Agency (IAEA) in Vienna to provide an improved international safeguards regime. In particular, he said that the Administration would seek agreement on requiring IAEA safeguards on all nuclear activities in a non-nuclear-weapon state as a condition for any significant new nuclear supply commitment.

Doubts about the efficacy of IAEA safeguards procedures, however, linger on — particularly in the light of evidence provided to the Senate Foreign Relations Committee last month by ex-IAEA inspector Mr Roger Richter.

The Department of Defense, at least,

shares these doubts. In an inter-agency report submitted to the National Security Council, some of which was leaked to the *Wall Street Journal* last week, the Pentagon expressed doubts about the "weakness of the IAEA as an international institution", complaining of the agency's "lack of an intelligence capability and the limits of its scope and jurisdiction", and warned against "undue reliance on the IAEA by those responsible for national security". Administration officials insist, however, that international efforts to bolster the IAEA, including efforts to develop effective regimes for international plutonium storage and improved cooperation for spent fuel management, must remain central to any global non-proliferation strategy.

One significant deviation from Mr Carter's approach was Mr Reagan's announcement that his Administration intended to use the supply of conventional arms to allies as a way of reducing the motivation to acquire nuclear weapons. This argument has already been used to justify the offer of a five-year military aid package to Pakistan.

Mr Reagan also announced that the White House would seek to persuade the Senate to ratify Protocol I of the Treaty for the Prohibition of Nuclear Weapons in Latin America, the so-called Treaty of Tlatelolco. This protocol, already ratified by the United Kingdom and the Netherlands, calls on nations outside the treaty zone to apply the "denuclearization" provision of the treaty to their territories in the zone.

The goal of the Administration's new policy, Mr Reagan said, was "to re-establish a leadership role for the United States in international nuclear affairs". He gave few clues, however, as to how the United States would punish countries which transgressed its guidelines, stating merely that his Administration would view a material violation of the Non-Proliferation Treaty and the Treaty of Tlatelolco as having "profound consequences for international order and United States bilateral relations". Similarly he said that the United States would "view any nuclear explosion by a non-nuclear-weapon state with grave concern".

David Dickson

UK universities complain to government

A last-minute attempt to persuade the British government to change its mind about the scale on which university budgets are to be reduced was undertaken last week by the Committee of Vice-Chancellors and Principals. A delegation led by Dr Albert Sloman, chairman of the committee and vice-chancellor of the University of Essex, urged on the Secretary of State for Education, Mr Mark Carlisle, that it would cost less to arrange for a slower contraction of the system than to foot the bill for buying out the tenure contracts of academics now likely to lose their jobs. Dr Sloman said that he and his colleagues had been encouraged to hear Mr Carlisle say that he "now understood the problem".

The vice-chancellors' case is simple. University budgets will fall in the next three years by about 15 per cent because of reduced public subventions, the further loss of students from overseas and because the numbers of domestic students are to be reduced. The result is that university staffs will also have to be reduced — the committee's estimate is that 3,000 academics and 4,000 others will have to leave British universities. The direct saving to the government in the next three years will be £400 million, but the cost of buying out tenured contracts could well be £250 million. So the government would save money if the pace of contraction were 2.5 per cent a year, not twice as much.

The estimate of the cost of breaking contracts stems from legal advice the committee has been taking in the past few weeks. Where an academic's contract with

a university assures employment until retiring age (65), premature firing would expose the university concerned to a civil suit. Compensation for breach of contract might range from £40,000 to more than £100,000, according to age, salary and the courts' estimate that the academic concerned would find another job. One complication to come to light is that only 80 per cent of British university teachers are covered by such cast-iron contracts.

Dr Sloman and his colleagues wrung their hands last week over the prospect of endless unseemly legal battles with former academic colleagues. Court cases could take three years to settle. Although technically individual universities would be liable for damages, the vice-chancellors are counting on the government to implement a promise by one of its ministers that no institution would be driven into bankruptcy.

The prospect of mutual assistance within the university system seems, however, to be fading. The pooling of financial reserves (now estimated at less than £75 million) is unlikely to be appealing to the best-endowed universities, usually dealt with leniently in last month's cuts. Schemes for finding jobs for displaced academics at other institutions are likely to founder, according to Dr Sloman, on people's likely calculation that a court settlement would be more advantageous than a job elsewhere. Reducing salaries, or switching to 10-month contracts, would require negotiations with the trade unions for which there is no time.

The actual scale on which redundancies will occur remains to be determined. The committee's estimate of 3,000 academic redundancies is identical with that given by Dr Edward Parkes, chairman of the University Grants Committee, to a parliamentary committee earlier this year. Ministers at the Department of Education and Science are plainly sceptical about such large estimates. But Lord Flowers, vice-chairman of the committee and rector of Imperial College, said last week that not a single university institution in Britain would be able to avoid some redundancies before the next academic year is out.

The committee's calculations also suggest that the consequences of the decreed reduction of student numbers will be more serious than had been foreseen. Total numbers of home students are to be reduced from a peak of 272,000 (expected in the coming academic year) to 249,000 in 1984-85. This, the argument goes, will require that student intakes should be reduced from 78,000 (in the coming year) to 70,000 in 1982-83 and thereafter.

Salford University

Industry advertises

British industry has come to the aid of the University of Salford, the institution singled out by the University Grants Committee (UGC) earlier this month for the largest cut in grant and student numbers. Earlier this week, three leading manufacturing companies joined with the university in announcing the creation of the Campaign to Promote the University of Salford — CAMPUS. To launch the campaign, nearly 200 companies contributed to the cost of an advertisement in three national newspapers yesterday extolling Salford's virtues and calling for further support.

The scale and spontaneity of the support from industry suggests that UGC in deciding where the cuts should fall, may indeed have paid too much attention to grants awarded to universities by research councils and not enough to research funded by industry. The universities that are to suffer the largest cuts in their incomes, Bradford, Aston and Salford, are chiefly technological institutions, noted for their attempts to forge links with local industries. The UGC has told Salford that by 1983-84 its grant will be 44 per cent less than in 1980-81 and that it will have to cut student numbers by about 30 per cent. Although the university's arts faculties will suffer most, science and technology too will be cut drastically.

GEC-Marconi Electronics, Ferranti and Ward & Goldstone Ltd of Salford are the three companies that have taken the initiative in setting up CAMPUS. They say that the University of Salford has provided them with a steady stream of appropriately qualified graduates — GEC has employed 250 in the past seven years — has fostered

successful technology-transfer between university and industry and provides useful refresher courses for experienced technologists and managers. The local authority has also given support, especially to a new science park which is being established near the university campus in an attempt to revitalize manufacturing industry in the north-west of England.

The campaign has two aims: in the short term to convince UGC of the error of its decision and to persuade it to reinstate some of the grant it is taking from the university; and in the long term to foster closer cooperation with industry, in the running of the university. The campaign, however, is not a fund-raising exercise, according to Professor John Ashworth, vice-chancellor designate of the university. Professor Ashworth, who is in the embarrassing position of being chief scientist at the Central Policy Review Staff, the government's think tank, until he takes up the vice-chancellorship in September, hopes that, if the campaign's short-term aim fails, he will be able to call on the industrial supporters of CAMPUS for advice in restructuring the university.

Precisely what action CAMPUS takes in persuading the government and UGC of Salford's merits remains to be seen and will to some extent depend on the response to this week's newspaper advertisements. The other aggrieved technological universities are also being invited to join in the battle.

Judy Redfearn

Carcinogen regulations

Cleansing solution

Washington

Tension between the scientific staff of the US Department of Labor's Occupational Safety and Health Administration (OSHA) and their new political bosses came to a head last week, stimulated by complaints from the European-based International Agency for Research on Cancer (IARC) about the interpretation of data on the carcinogenicity of formaldehyde.

The dispute arose from a letter written by Dr Peter Infante, head of OSHA's Office of Carcinogen Identification and Classification, to Dr John Higginson, director of IARC, which queried the findings of an IARC working group that there were only "limited" data providing evidence of the carcinogenicity of formaldehyde on laboratory animals. Also included was a circular published last year by the National Institute of Occupational Safety and Health (NIOSH) describing formaldehyde as a carcinogen on the basis of various uncompleted studies with rats.

Dr Higginson complained that Dr Infante's letter appeared to "cast aspersions on the scientific integrity and objectivity" of the members of the IARC working group. And shortly afterwards Dr Infante was told by OSHA that he was

being fired for "insubordination" in sending his letter on official notepaper giving what the agency now describes as a purely personal opinion, as it is no longer prepared to endorse the conclusion that formaldehyde is, in fact, a carcinogen, and has withdrawn its co-sponsorship from the NIOSH bulletin.

Dr Infante's case is being seen as a symbol of the new Administration's determination to limit the powers of an agency previously accused of imposing a massive burden of regulation on the private sector. Already the director of NIOSH, Dr Anthony Robbins, has been dismissed by Mr Richard Schweiker, Secretary of Health and Human Services, as a "political activist", and the Administration has also announced its intention to move NIOSH administrators out of Washington, a step which many agency officials feel is designed to weaken their effectiveness. Last Thursday the head of OSHA, Mr Thorne Auchter, was confronted at an angry hearing by Congressman Albert Gore, chairman of the House Science and Technology Committee's subcommittee on oversight and investigation, who described Dr Infante's firing as unacceptable political pressure on the work of scientists.

On the previous day, several high-ranking government scientists, including Dr Vincent DeVita, director of the National Cancer Institute, had agreed that data produced last year by the Chemical Industry Institute for Toxicology provided a "sound scientific basis" for suspecting that formaldehyde was carcinogenic in humans.

At the Thursday meeting Mr Gore produced a memorandum, written to Mr Auchter by his special assistant for regulatory affairs, describing the latter's meeting with representatives of the formaldehyde manufacturers. At the meeting doubts were raised about the data on which the previous administration had based their description of formaldehyde as a carcinogen, doubts which Mr Gore suggested formed the basis of the decision by Mr Auchter to withdraw sponsorship of the circular issued jointly with NIOSH last December.

Dr Infante's immediate boss, Dr Bailus Walker, in his letter of dismissal said that OSHA "lacks confidence in the data" on which the circular, which has no regulatory impact but is meant primarily for information, was based. Dr Walker told Mr Gore that he had dismissed Dr Infante "on the advice" of his boss Mr Auchter.

Mr Auchter agreed that his decision that the carcinogenicity data were inadequate to support the conclusions of the circular, was made without consulting any of the agency's scientists. However, he insisted to Mr Gore that his lack of confidence was based solely on the use of the data for regulatory purposes, as conflicting data also existed.

Dr Infante's dismissal is now the subject

of litigation between himself and the agency, a fact which Mr Auchter initially used as a reason for not providing detailed comments on the case to the investigations committee. However, under pressure from the Congressmen, who presented legal advice that the hearings would not prejudice any later trial, Mr Auchter shifted his stance — and went on to deny that he had ordered Dr Infante's dismissal, directly contradicting the sworn testimony given by Dr Walker.

Dr Walker, who told the subcommittee members that "the data suggested that formaldehyde was a potential carcinogen and should be treated as such", has now resigned from OSHA to take up the position of director of public health for the State of Michigan.

The subcommittee has yet to announce the conclusions of its investigations. Indeed one minority member, Republican Congressman Robert Walker, sharply defended OSHA's actions on the grounds that Dr Infante had broken federal rules in representing his own scientific opinion as that of the agency. Mr Gore, however, was in no doubt "that the formaldehyde industry had "engineered a decision in the agency to change OSHA's view on the scientific data" and that this was behind the decision to fire Dr Infante.

David Dickson

Local DNA guidelines

Boston strikes out

Boston

In what could become a prototype for American cities seeking control of recombinant DNA research, the Boston City Council has passed a law regulating research at universities and commercial companies. The city ordinance follows community activism by residents of the Mission Hill district of Boston, who are concerned about the \$50 million grant by the Hoechst chemical company to the Massachusetts General Hospital and by the leasing of empty hospital space in their neighbourhood to Genetics Institute Inc., the newly formed genetic engineering company.

The city council hearings of the past month have actually been a repeat of events in nearby Cambridge eight years ago, when recombinant DNA research was entirely new. But on this occasion, there was less open conflict between citizens and university officials than in Cambridge.

The new law requires compliance with National Institutes of Health (NIH) standards but there are also further local restrictions which have been added since work began on the original proposal in late May. As well as assuring strict conformity with NIH guidelines, the ordinance demands:

- "Timely response to guideline amendment and permit applications in accordance with good governmental

practice".

- Research proposal not subject to NIH guidelines should receive council-administered permits.

- Broadening and restructuring of the Boston Biohazards Committee — an area regulatory organization — which will now serve as an advisory board to the commissioner of the Boston Department of Health and provide an annual report to the city council.

- Opening of normally confidential employee health records for "regulatory or public health study purposes".

- Institutions performing recombinant DNA research should monitor the health of their employees and the institutional responsibilities in this area should be "reasonable and related to potential risks".

- Costs of monitoring to be reimbursed to the city by the regulated institutions.

The final version of the ordinance left out the harshest requirement of the original proposal — that institutions should perform regular effluent monitoring and the testing for live organisms in the city sewer system. This was dropped because it is not technologically feasible.

In the past, local universities have agreed that guidelines of some sort would be helpful but have opposed the introduction of laws requiring compliance with official regulations, arguing that universities should set their own standards. But campus officials are generally pleased with the outcome of this debate and confident that they will easily meet the provisions.

The new ordinance will run for five years and is renewable. Several other cities in the Boston area and elsewhere in the United States have begun to review their own proposals for regulation as commercial companies are proliferating.

Michael D. Stein

Aspartame sugar substitute

New court overruled

Washington

It had been described as the first official attempt to resolve a complicated dispute over the safety of a new food additive by using a so-called "science court", with evidence on both sides being presented to a panel of three outside scientists. But last Wednesday the US Food and Drug Administration (FDA) overruled the verdict of its Scientific Public Board of Inquiry, reached after hearings held in January and February last year, and approved the marketing of a new low-calorie sweetener, aspartame.

Permission to market the sweetener had first been requested from FDA by its manufacturers, G.D. Searle, seven years ago. Initially FDA had agreed; but in the light of reports from a scientist at Washington University, St Louis, that the sweetener could produce brain lesions when fed to laboratory animals — and the general

concern that accompanied the decision to ban cyclamates in 1970 — permission was withdrawn the following year pending further studies.

Doubts about the validity of animal studies conducted for Searle to generate the data needed for new drug approval were discounted after two years of independent auditing of the studies. FDA then turned to the brain lesion claims, which were examined by a three-person team headed by Dr Walle J.H. Nauta, professor of neuroanatomy at the Massachusetts Institute of Technology. In their report, issued last October, the scientists said the data shown to them did not support the suggestions that aspartame might kill clusters of brain cells or cause other types of brain damage.

However, the "science court" also raised doubts about whether the reports of brain lesions could be completely discounted on the grounds that the tests had been carried out at doses far higher than those humans would normally experience. It recommended that marketing approval be withheld until further long-term animal tests had been carried out to rule out any possibility that aspartame could result in brain damage.

This conclusion was bitterly contested by Searle, already sitting on a stockpile of 300,000 lb of the sweetener, with a market value of over \$25 million. The company claimed that the three scientists' conclusions made "significant errors" in dealing with issues of tumorigenicity; and that they had "failed to employ biological and statistical principles that would have provided guidance in assessing the potential carcinogenicity of a compound".

Searle pointed out that the sweetener is already being marketed in France, Belgium and Luxembourg, and that it has also been approved for use by the Joint Expert Committee on Food Additives of the Food and Agriculture Organization, and the World Health Organization.

The new FDA commissioner, Dr Arthur Hayes, now seems to have accepted Searle's arguments. Following the conclusion of the agency's Bureau of Foods that the "science court's" concerns were unfounded and that the sweetener would be safe even at the "highest conceivable levels" of consumption, he has agreed that it should be approved for use as a sugar-substitute and food additive, although not yet for soft drinks.

David Dickson

Environmental lead

Playing safe

Britain's health and environment departments seem to have won a victory behind the scenes in the government's decision last month to reduce the lead content of petrol from 0.4 to 0.15 g per litre. Other government departments concerned about the financial effects on the car and oil refining industries finally gave

way last May to the arguments that lead from car exhausts could damage the health of children in the inner cities. The environment and transport departments, however, supported the decision chiefly as a way of countering some of the increasingly fierce objections to building new roads.

The decision on lead in petrol came as a surprise just one year after a report commissioned by the health department concluded that there is no proof of a link between low blood-lead levels in children and impaired mental development. That report, prepared by a working party under the chairmanship of Professor P.J. Lawther of St Bartholomew's Hospital, was stimulated by research in Germany and the United States which implicated blood-lead levels as low as 35 µg per dl, now accepted as the maximum permissible by the European Commission. Although the working party concluded that these and other studies were equivocal, it recommended that steps be taken to reduce lead in food and water, seen as major sources, and air, a less significant source.

The Department of Health, however, seems to have persuaded the Department of Energy and the Treasury to act on petrol on the basis of evidence not covered in the Lawther report. A study by Dr R.G. Lansdown of the Great Ormond Street Hospital and Dr William Yule of the University of London Institute of Psychiatry of children in the Greenwich area of London is thought to have shown a correlation between low blood-lead levels commonly found in the British population and a slight impairment in IQ. That study, although made known to government officials, has not yet been published because of difficulties in interpreting data. Dr Yule now expects it to appear in the October issue of *Developmental Medicine*.

Other evidence which seems to have weighed in the decision includes the findings by the Greater London Council of exceptionally large quantities of lead in dust in some inner city playgrounds, research at ISPRA in Italy which suggests that 25–40 per cent of the total body lead burden may be contributed by lead in petrol and a critique of the Lawther report prepared by Professor D. Bryce-Smith and Dr Robert Stephens for the Conservation Society. The Conservation Society's document complained that the Lawther report did not pay sufficient attention to animal studies and that it underestimated the contribution of airborne lead by failing to allow for the fact that much of the lead in food and dust settles from the air.

The Department of Health seems to have persuaded the government to play safe by ordering the reduction. The search is still on, however, for better evidence of the possible effects of low lead levels on health. Dr Lansdown and Dr Yule are at present engaged on another study of inner city children and are discussing the possibility of a third, more extensive study with the Medical Research Council. **Judy Redfearn**

UK defence research

Slimming down

The Royal Aircraft Establishment at Farnborough, which with its several outstations is the largest of the in-house research stations run by the British Ministry of Defence, seems also to be the first in line for substantial reorganization. In the past year, the total staff of the establishment has fallen from 4,500 to 3,800, largely as a result of natural wastage (principally retirement) made possible by the top-heavy age distribution at the laboratory, itself a consequence of rapid recruitment during and immediately after the Second World War. But the remaining staff at the laboratory are now anxious that attrition has a lot further to go.

The establishment is the most conspicuous of the defence research establishments considered by the internal government review under Lord Strathcona, whose report has not been formally published but has instead been placed in the library of the House of Commons, where its presence is almost certain to have the most damaging consequences. In respect of Farnborough, the report recommended that further consideration should be given to the need for maintaining three airfields (at Farnborough, Bedford and Boscombe Down), a balloon station (at Cardington), three missile ranges and a variety of wind tunnels that (in Lord Strathcona's view) could well be hived off to private contractors.

At a meeting at Farnborough on 4 March, the director of the establishment, Mr T.H. Kerr, told the staff that the future of these offshoots was being considered by a series of working parties, and that decisions would be made later in the year. The most obvious possibility of change is that missile ranges may in future be managed by private contractors (as is to a large extent already the case at the Welsh firing range at Aberporth). But there are also to be rearrangements of the internal divisions at Farnborough (leading to the loss of relatively senior staff) and the future scale of operations at the establishment has not yet been determined, which is why some members of staff fear that their director's promise (on 4 March) that reductions could be accomplished by retirement, early retirement and other wastage (as well as the transfer of some staff to private contractors) may not be feasible.

The chief source of anxiety in the past few weeks, however, is strictly speaking hardly relevant — the steps being taken by private interests in case the Farnborough airfield is eventually put up for sale. It seems to have been decided that, if any of the three airfields is to be sold, the sale of Farnborough would cause the least damage even though the establishment would wish to retain the right to mount the biennial air show of the Society of British Aerospace Companies on the site. The bid to purchase

New Forest flutter

In the name of science, lepidopterists have for many years, chloroformed butterflies to death and displayed them in glass cabinets. Now many of those butterflies are increasingly imperilled by loss of habitat, killing by insecticides and other hazards of the twentieth century. A cautious welcome then, for Britain's very first public "butterfly farm", which opened yesterday (22 July) in the New Forest. It's not being run for the good of science though, simply as a tourist attraction to bring in money to an estate at Ashurst, near Southampton.

There should be more than 1,000 butterflies at Ashurst if the breeding programme is successful, flying freely in a glasshouse covering 6,750 square feet.



Now in Hampshire — the American monarch

Over 50 different species will be on view, mostly colourful imports from the tropics, including the North American monarch, *Heliconius* from South America, swallowtails from South-East Asia, and the Camberwell beauty.

Like all would-be "tourist traps" the farm boasts a gift shop. Books on butterflies will be on sale, of course, but so too will live caterpillars and pupae, presumably to encourage butterfly farming as a hobby. Traditionalists are also catered for, they can buy mounted butterflies.

None of this is likely to do any direct good to the average butterfly in the street, or field. But the "farm" owners reckon that by increasing the public's awareness of what they call "these delicate creatures", life will gradually improve for what's left of Britain's butterfly population.

Charles Wenz

the airfield is based on the hint in the Strathcona Report that the airfield would be suitable as a base for business flying, but will not be considered until all the working parties considering the future of the defence research establishments have had their way.

Last week, at another staff meeting, Mr Kerr had little extra to say. Discussions have begun with potential operators of the parts of the establishment to be hived off, but firm proposals are not expected until early next year. Bidders for these contracts (among which British Aerospace is conspicuous) are being asked to take over existing members of the staff — and unions such as the Institution of Professional Civil Servants are looking for an assurance that their members' terms of employment will not be adversely affected.

French energy policy

The great escape

There were two striking omissions from the roster of nationalization proposals announced by the Prime Minister of France, M. Pierre Mauroy, recently — Framatome and Novatome, the companies which between them account for almost the whole business of constructing conventional and fast breeder reactors in France. The exceptions are remarkable because the Socialist Party had announced before the election that all industries concerned with the nuclear fuel cycle would be nationalized.

M. Mauroy was, however, quite clear in what he said: eleven groups would be nationalized, not one more nor one less. The omission of the nuclear industry is another indication that the new government does not intend seriously to disturb France's growing lead in nuclear technology. Mauroy explained that the companies to be nationalized are included in his list not for doctrinal reasons but to improve their competitive performance — as has been done with the car maker Renault. (In fact the new Minister for Industry, M. Pierre Dreyfus, was previously managing director of Renault, so his appointment has somewhat comforted industrial opinion.) Thus the immunity of the nuclear business amounts to a tacit recognition of its independent commercial success.

The grand debate on energy also begins to look less of a confrontation than a few weeks ago. It is expected now to take place in the National Assembly in November — and instead of focusing on nuclear power it will look at all aspects of the energy question, a difficult matter in what will be a packed legislative session. Parliamentary and specialist groups have been set up to investigate separate issues.

Anticipating the debate, M. Mauroy has seen fit to announce the broad outlines of French energy policy. The two priorities will be to develop indigenous fossil fuel production, insofar as that is economic (taking account of world prices) and to pursue a significant nuclear programme.

Mauroy did not mention renewable sources or even conservation, and his remarks on fossil fuel production are taken to imply caution in the further development of France's costly coal compared with the cheaper Australian product. That leaves, of course, nuclear power as the central plank of his energy policy.

On this reading, the government must arrange that the November energy debate will lead to a vote to continue to build new pressurized water reactors. (New starts are temporarily suspended.) The construction of Superphénix, the commercial demonstration fast breeder reactor, continues, and a decision on a second one can be comfortably left for a while.

Robert Walgate

Mongolia's jubilee

Following the lamas

The Mongolian People's Republic this month celebrates the diamond jubilee of its foundation on 11 July 1921. Included, *de facto*, in the anniversary is the Mongolian Academy of Sciences, since although formally inaugurated only in May 1961, it originated, in the words of Dr B. Shirendyb, president of the academy, from "a small group of scientific workers" who banded together immediately after the establishment of the Republic to raise the scientific standards of the country.

Not that science had been entirely neglected in pre-revolutionary Mongolia. An astronomical observatory had been founded in Ugra (now Ulan Bator) in 1779, and a flourishing medical tradition was preserved in the lamaseries. Nevertheless, in the early years of the new state, the major task was the translation of basic scientific texts from Russian and other European languages. And before even this could be done a more fundamental problem had to be solved of replacing the picturesque but impractical traditional script by a modern alphabet.



Astronomical observatory Mongolian style

Inevitably, present day research in Mongolia is largely directed towards the needs of the economy. During the past five years, the various institutes of the Academy of Sciences carried out 369 research projects, which should ultimately save the economy more than 108 million tugriks (£18 million). Research included the use of methods of nuclear physics in surveying the country's deposits of copper, molybdenum and tungsten, mapping of seismic zones, the extraction of pharmaceuticals from traditional medicinal herbs, and the development of new strains of wheat and breeds of sheep.

International help has not been lacking. The plan to establish a 5 million hectare nature reserve in the Gobi Desert is receiving considerable assistance from the United Nations Environmental Programme, and Comecon countries have some 60 joint research projects in operation with Mongolia. One piece of research, not yet covered by a formal agreement but watched with considerable interest by Mongolian scientists, is the work of Dr Tibor Farkas at Szeged

(Hungary) on the biophysics and biochemistry of frost damage. The Mongolians hope that this work will result in the development of an anti-frost spray for spring wheat, which is at considerable risk in their harsh climate.

Among the facilities that Mongolia has to offer its international collaborators is the Gobi Desert — a valuable hunting ground for Comecon expeditions in search of fossils of dinosaurs and dinosaur eggs.

Vera Rich

Belgian scientific institutions

Arresting decline

Brussels

It has long been felt in Belgium that some of the country's most renowned scientific institutions are falling into decline. Disaffection is so great that last spring the staff of eight major institutions resorted to strikes to draw attention to their grievances. Now at last the government has decided to do something.

A committee of wise men has been given the task of finding ways to unravel the administrative snarls which are held responsible for the problems. The eight institutions under review are the Royal Library of Belgium, the National and Provincial Archives, the Royal Belgian Institute of Natural Sciences, the Royal Museum for Central Africa, the Royal Belgian Observatory, the Royal Belgian Meteorological Institute, the Belgian Institute of Aerospace and the National Centre for the Production and Study of Microbiological Substances.

The problems of these institutions are uniquely Belgian. When the constitution was revised in 1970, it was decided to keep them as national bodies. The conflict between the Flemish and Walloon (French-speaking) communities in Belgium has led to a virtual duplication of all administrations and much else. The education system is completely divided along linguistic lines. As national bodies, the eight institutions in question fall foul of the system and do not benefit from the patronage of either the French or Flemish education ministers.

Every decision has also to be approved by three or four ministries and with a change of government every few months, the national institutions have found it very difficult to get anything done. Most of the directors of the institutions have left in disgust at the administrative confusion.

The committee is due to make its first report within the next three months, but the recommendations are bound to involve more drastic cures than simple administrative pruning. Some of the institutions with more active research departments are sure to be merged with universities. And the worrying size of Belgium's public-sector deficit may prompt the committee members to recommend that parts of the institutions should be axed completely.

Jasper Becker

CORRESPONDENCE

Radical writings

SIR — Dr Turner's letter (*Nature* 4 June, p.374) makes the disturbing claim, citing the exchange between Dawkins and myself as evidence, that "the radical scientific opposition to racism requires a denial that there is any genetic variation of any significance from place to place within the human species". I wonder what on Earth he has been reading which provides support for such an assertion? As someone who has been reading, writing and teaching in this area for some years now, I can't remember ever seeing such a claim, and I would be interested if he could produce a single statement from the writings of "radical" biologists since the sociobiology and IQ debates started in the late 1960s which could support his allegation.

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STEVEN ROSE

Australian 2,4,5-T

SIR — Since you published our letter¹ on "Antipodean 2,4,5-T", a sample of the herbicide imported into Australia has been brought to our attention. It had been submitted to the Australian Tariff Board by the importer as a sample of a very large consignment on which lower tariffs had been sought. It was recently analysed by the West Australian Government laboratories and found to consist essentially of the isobutyl ester of 2,4,5-T, and to contain 19 mg per kg of 2,3,7,8-TCDD on an "as received" basis, and 26 mg per kg on a 2,4,5-T basis. (The level of TCDD (2,3,7,8-tetrachlorocyclohexa-*p*-dioxin) in 2,4,5-T considered acceptable in Australia at the time was 0.1 mg per kg.)

Evidence given before the Tariff Board inquiry suggests the material may have been produced from fire-damaged potassium trichlorophenolate (KTCP). [A synthetic route to 2,3,7,8-TCDD is through the heating of KTCP^{2,3}.] A letter from the Singapore High Commission (Australia) has confirmed that a fire occurred in the premises of the importer's Singapore company, and pointed out that (1) there were reports of chloracne in the factory workers and (2) after the fire, the neighbouring factory complained of symptoms in its workers. The importer himself described this "fire damaged" material as of "good value"⁴, and so one assumes that it entered commerce in Australia.

All of this makes more pressing our concern for Australia's epidemiological inquiry into Vietnam veterans' claims. Use of 2,4,5-T with such high levels of TCDD can prejudice the controls in this study. It will also have implications for ordinary civilian usage.

We hope that one result of our work will be that the Australian authorities will increase their surveillance of chemical imports.

PETER HALL
BEN SELINGER

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Conservation areas

SIR — It would seem injudicious for Dr Ratcliffe to claim¹, as his guarantee of rightness, the wisdom of Parliament when a minister of the Crown can say², after receiving the advice of the Nature Conservancy Council (NCC) "I welcome the international recognition that it is totally unacceptable for species to be allowed to disappear", though this is quite in keeping with the NCC's concern to safeguard Sites of Special Scientific Interest (SSSIs) "in perpetuity". If the NCC has not got the financial resources to provide invariable, immediate and adequate compensation to the owners of SSSIs, how will their perpetual preservation policy prevent glacial readvance over the Black Wood of Rannoch, or pluvial destruction of the Pennine limestone pavements, or, perhaps, provide fall-out shelters for Dartford warblers?

I suppose that one must expect if one has the temerity to express an unfashionable point of view that one will be misunderstood accidentally and deliberately^{3,4}, so may I make it clear that I am not advocating the wilful destruction of anything, organism or habitat, but I do feel that anyone, acting as a scientist, needs a better excuse than a personal predilection for the rare, the exotic, or the *status quo* before he asks people to forgo the benefits of cultivating their gardens or puts others to the national expense of compensating them for their abstinence. I had been led to believe that science was an organized body of knowledge concerned with the physical world as we find it, static or dynamic as the case may be, that scientists were by their training perhaps better equipped than most to make reliable predictions of the likely outcome of the processes which they observe, but that as scientists they have no claim to superior knowledge or wisdom in prescribing what goals or courses of action are desirable on moral, political or economic grounds. It is against this assumption of authority, which is more than hinted at in Dr Ratcliffe's last sentence, that I am railing.

COLIN MUIR

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Israeli raid

SIR — I was unpleasantly surprised to read the leading article in *Nature* entitled "Making Israel atone for Tamuz" (18 June, p.523). I and many others of your readers are of the view that *Nature* is a scientific journal, which justifiably includes articles on scientific or educational policy in addition to the research material which it publishes. However, this was an article of a purely political nature, written about a very sensitive issue without any backing of specialized knowledge. It was in my view an unwise decision to publish such an article in *Nature* and a dangerous precedent.

It is, of course, impossible to enter into a full discussion with you on the reasons for Israel's decision to bomb the nuclear plant in

Iraq, but there are certain facts which have been ignored in this article, which should be brought to your attention.

(1) There is a great deal of evidence that Iraq has ambitions to acquire a nuclear weapon and it has built up its military power in the last few years to an alarming magnitude. As this country is at war with Israel, having consistently refused to enter into any peace negotiations, this must be a serious threat to the security of Israel.

(2) To ask Israel to accept the report of the UN International Atomic Energy Agency is expecting a lot, in view of the attitude of the United Nations and its agencies towards Israel in the past. If Israel is to survive it must depend on its own intelligence assessments. This is exactly what it did in this case, and it acted before it was too late.

(3) The article ignores the fact that Israel has never refused to enter into peace negotiations with its Arab neighbours. Except in the case of Egypt these countries have not only refused but quite openly proclaimed their ambition to destroy the State of Israel; Iraq has been one of the leading advocates of this policy. Under these circumstances how can one expect Israel to enter into a "Non-Proliferation Treaty"?

ANNE BELOFF-CHAIN

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Forbes, not Wallace

SIR — In his review of a recent symposium on biogeography, Colin Patterson regards A. R. Wallace as the inventor of "biogeography in the accepted sense", and gives the impression that he thinks very little of the scientific competence of Charles Darwin in this context (*Nature, News and Views* 25 June, p.612).

I refrained from joining in the previous arguments in your columns about cladism, evolution and Darwin, but in this instance I must protest that in enthroning Wallace and demoting Darwin, Dr Patterson is overplaying his hand and ignoring the earlier literature. Darwin was well aware that the continents were not immutable (see the sketch of 1842 and the essay of 1844), and that previous epochs may have had land connections different from those of the present. It is incorrect to claim that evolutionary hypotheses based on unchanging continents dominated biogeographical thinking up to the 1960s, when Wegener's views were finally accepted by orthodox geophysicists. Many of the published biological arguments of the period were about the extent and timing of the land and sea connections, not whether they existed.

Darwin was also aware of contemporary studies of geographical distribution, such as the excellent review of Edward Forbes, not mentioned by Dr Patterson, published in the first volume of the *Memoirs of the Geological Survey*, 1848. In this article Forbes clearly stated an hypothesis for a previous land connection between the Iberian peninsula and Ireland. If anyone is to be selected as the father of biogeography it should be Forbes.

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NEWS AND VIEWS

Ocean currents and wind: how well can the connection be made?

from Charles C. Eriksen

A recent issue of the *Journal of Geophysical Research* (86, C3, 20 March 1981) contains two articles which serve to demonstrate the wide gulf between observations and models of wind-driven ocean response. The paucity of reliable upper ocean measurements and suspension of Ekman dynamics make the situation even worse near the equator. One article, by S.G.H. Philander and R.C. Pacanowski, describes the results of a numerical simulation of the response of a stratified equatorial ocean to periodic wind forcing at periods from days to years. The other, by R.A. Weller, describes current profiles made at mid-latitude from a drifting research platform. While Philander and Pacanowski make rather detailed predictions of oceanic response, Weller finds that Ekman theory, a cornerstone of dynamical oceanography (W.V. Ekman in 1905 predicted current profiles which spiral and decay with depth through a balance of frictional and rotational forces in response to wind stress at the surface), fails to account for the rather weak decay of shear away from the surface which he observed.

The problems of measuring in the upper ocean are formidable. It is only recently that instruments have been developed which succeed in removing the contaminating effects of surface waves on current measurements. This success is achieved by utilizing linear sensors which can deal with currents which are not confined to the horizontal plane. The time scales of wind forcing and ocean response make sequential measurements at one or more fixed locations an attractive method of examining ocean physics. These eulerian descriptions of the flow are often collected from current meters tethered at fixed depths along a deep sea mooring. Weller used newly developed current meters in a profiling mode, raising and lowering them by winch from the uniquely stable Research Platform *Flip* (a giant spar buoy resembling a submarine tipped on end, which pitches and rolls hardly at all). Since *Flip* drifted modestly, the measurements are not eulerian, but their excellent accuracy allows records of current shear to be compared with time-dependent Ekman

models. Although the observed changes in phase with depth are modelled by simple Ekman dynamics at most frequencies, the amplitude structure is not. The discrepancy ultimately seems to be nonlinear. Turbulence produces apparent diffusivities which greatly exceed molecular values. The spatial structure of eddy viscosity (turbulent diffusion of momentum) is poorly known, as is the role of larger-scale nonlinearities in the flow. These two unknowns keep upper ocean response to wind stress in the class of 'fascinating unsolved problems' in oceanography and promise to do so for some time in the future.

Near the equator, Ekman theory breaks down because the horizontal Coriolis forces associated with the Earth's rotation vanish. There is no satisfactory theory for the planetary boundary layer at low latitudes; most theories use a body force formulation (stress distributed *a priori* in a slab of water) to circumvent this problem. This arbitrary linkage is used to provide a forcing mechanism, to which the response by large-scale ocean dynamics is sought. Numerical models are often the only means by which the complicated effects of density stratification, friction, nonlinear effects and ocean boundaries can be considered together. In the equatorial oceans it is clear that all of these effects are important. Everyone seeks simple force balances, and discarding one or more of these effects in favour of the others is a popular method of equatorial theorists. Unfortunately, largely for technical reasons, there is relatively little guidance from observation for the correct choice in a given situation. Numerical studies also require data taken at discrete intervals to keep computations manageable (even the largest computing facilities can be easily overburdened by the size and density of calculations demanded by modest models of ocean circulation). The resulting simulations are not altogether realistic, but at least describe the

mechanisms at work in different flow regimes. Philander and Pacanowski state various results about how the ocean responds to wind forcing, but it should be remembered that these are the results of a model study of a numerical ocean which one cannot be sure behaves exactly as the real oceans do.

The results Philander and Pacanowski report are an example of what can be said about oceanic response from an incorporation of the general effects, but not the details, of the mechanism by which wind energy is transferred to the ocean. They highlight the unique character of equatorial oceans — that it is possible to have a Kelvin wave trapped to the equator which can carry energy eastwards, in addition to equatorial analogues of the long Rossby waves at mid-latitude which carry energy to the west. Philander and Pacanowski identify three ranges of period: (1) at the short periods (a few days) strong currents are not generated, but rather a spectrum of equatorial waves; (2) at intermediate periods (weeks, months) upper ocean currents are generated primarily which reflect off the basin boundaries in a complicated fashion; and (3) at very long periods (greater than several months to a year or more, depending on the basin size) steady-state response is achieved. The nonlinear nature of the problem causes Rossby waves to be modified by the mean currents at low frequencies. Also, rays along which energy travels have shallow inclinations at long period, so that the deep ocean can only contain wave energy which is the result of one or more reflections off the basin ends. There are very few measurements by which any of the predictions of the model can be tested; however, the general contrast between observations in a small basin and a large one (Atlantic versus Pacific) tends to confirm the predictions of the model. It is a big job to observe enough of the ocean to really test these models in even the most rudimentary way. At this point large-scale theories are far more sophisticated than data. Observations are needed to verify the assumptions upon which the models rest, as well as the general model results.

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Migrating nitrogen atoms in diamond

from Gordon Davies

By crystallographic standards gem quality diamonds are highly impure. Most of them contain nitrogen at about one part in a thousand — some 10^{20} atoms of nitrogen per cubic centimetre of diamond. Significant colouring is usually produced in a crystal when it contains about 10^{17} impurity atoms per cubic centimetre, but natural gem diamonds are usually colourless because the nitrogen atoms have aggregated into pairs, each pair replacing two carbon atoms. The paired form does not absorb visible light as its absorption bands lie in the ultraviolet part of the spectrum. However, if even a small fraction of the nitrogen is present in the simplest form, as isolated nitrogen atoms with each atom replacing one carbon atom, then an unattractive brown colour is produced. Most synthetic diamond is of this type and is unsuitable for use as gem stones.

In nature many diamonds are also found in which the nitrogen aggregation has gone further, producing aggregates of three or more nitrogen atoms (see Fig. 1). Diamonds like these are often of considerable commercial value as gem stones, for in a triangular arrangement the nitrogen can absorb near-ultra violet radiation and emit the energy in the visible part of the spectrum, enhancing the brightness of the diamond in a whiter-than-white effect.

In the early 1970s Soviet science journals reported that synthetic diamonds grown at higher temperatures and pressures than usual had a small amount of their nitrogen in the aggregated forms typical of natural diamonds, although most of the nitrogen was still scattered through the lattice as isolated atoms. A few years later, at the General Electric Corporate Research and Development Laboratories in Schenectady, New York, R.M. Chrenko, R.E. Tuft and H.M. Strong observed substantial aggregation of the nitrogen in some of their own synthesized diamonds when the diamonds were heated to about 2,000K for 30 minutes. In one diamond they converted three-quarters of the isolated nitrogen atoms into aggregated forms. The technique was published in *Nature* (270; 141, 1977) and protected by patent (British Patent specification 1578987).

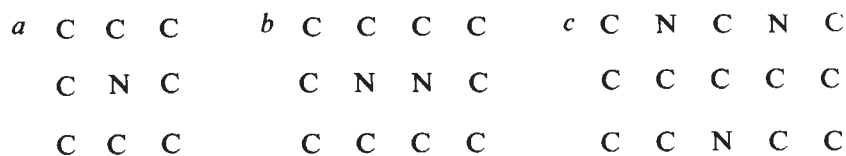
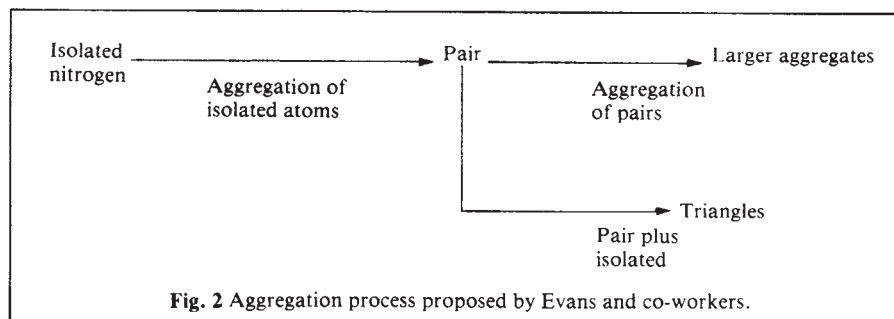


Fig. 1 Highly schematic diagrams of the smallest nitrogen structures in diamond: *a*, the isolated nitrogen atom, *b*, the nitrogen pair, *c*, the nitrogen triangle. In the nitrogen triangle, the N atoms share one common C atom as the nearest neighbour; they are not bonded directly together.



The possibility of improving the colour of synthetic diamonds may appear to have commercial implications for the gem industry. In practice, however, the cost of growing gem-sized diamonds is prohibitive, and to convert any nitrogen in the diamond requires a further heat treatment at the aggregation temperature of 2,000 K. The processed diamond would still not be the same as a natural diamond. Inclusions trapped inside a synthetic diamond are metallic, from the growth environment, and are easily distinguished from natural inclusions. In the world of gems, a colour-improved synthetic diamond would still be regarded as inferior to a natural stone and would be priced accordingly. Nor, in view of the recent announcement of the massive diamond find at Smoke Creek in the north of Australia, is there likely to be a scarcity of natural diamonds in the foreseeable future.

The value of the aggregation experiments is that they provide a tool for studying the geology and physics of diamond. At Reading University, B.P. Allen and T. Evans (*Proc. R. Soc. A* 375; 93, 1981) have shown that in aggregation, isolated nitrogen atoms first form pairs and then migrate to produce the larger aggregates (see Fig. 2). The longer the diamond is held at a high temperature, so the state of aggregation in any diamond gives some idea of the length of time it has been heated. The temperature at which natural diamonds grew can be measured independently from the chemistry of their inclusions; for all diamond sources a temperature of about 1,450 K is indicated (J.J. Gurney and B. Harte *Phil. Trans. R. Soc. A* 297; 273, 1980). Extrapolating the

laboratory results of Chrenko *et al.* down to this low temperature, Allen and Evans estimate that those rare natural diamonds containing only isolated nitrogen atoms must have been ejected to the surface of the Earth within 50 years of their growth — a very short time scale geologically.

The accuracy of this estimate is currently unknown because of a complication discovered by A.T. Collins at King's College, London (*J. Phys.* C11; L417, 1978). The speed at which isolated nitrogen atoms can migrate is increased if some of the carbon atom sites in the diamond are vacant. A nitrogen atom next to a vacancy has more room to manoeuvre in the crystal, leading sometimes to a 50-fold acceleration of the aggregation process (A.T. Collins *J. Phys.* C13; 2641, 1980). Collins has also suggested that the original measurements of Chrenko *et al.* may have overestimated the mobility of the isolated nitrogen atoms. Nevertheless, when the migration parameters of the nitrogen atoms are known better it will be possible to fix the time a diamond has spent deep in the Earth.

The nitrogen aggregation experiments have recently cleared up one long-standing problem of diamond research. Many natural diamonds contain two-dimensional defects, with diameters of the order of tens of nanometres, lying in their cube planes. These 'platelets' only occur when the diamond contains a large concentration of nitrogen. During the 1960s it was generally assumed that all the nitrogen was tied up in the platelets. Then E.V. Sobolev, V.I. Lisoivan and S.V. Lenskaya (*Sov. Phys. Dokl.* 12; 665, 1968) showed that there was no simple correlation between the number of platelets in a diamond and the concentration of nitrogen it contained. During the 1970s it was found that the optical properties of diamonds could be satisfactorily explained if the platelets were simply ignored, and the nitrogen assumed to exist only in small aggregates like the pairs of nitrogen atoms. But now, T. Evans, Zengdu Qi and J. Maguire (*J. Phys.*

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C14; L379, 1981) have produced platelets in a synthetic diamond solely by heating at 2,700 K for 4 hours and at 2,800 K for 2 hours. Initially, the diamond contained about 10^{20} cm^{-3} isolated nitrogen atoms. After the heat treatment some 10^{15} cm^{-3} platelets with diameters of 20–50 nm were produced — equivalent to about $2 \times 10^{19} \text{ cm}^{-3}$ defect atoms — as well as the other aggregated forms of nitrogen which accounted for some 10^{20} cm^{-3} nitrogen

atoms. The experiments can only be understood if the platelets are made of nitrogen. The apparent discrepancy in the numbers above is expected since the distribution of nitrogen in synthetic diamonds is inhomogeneous.

The picture emerging for the 1980s of nitrogen in diamond is that it is present in both the platelets and in the atomic-sized structures. Key experiments are required to complete the picture: accurate calibrations

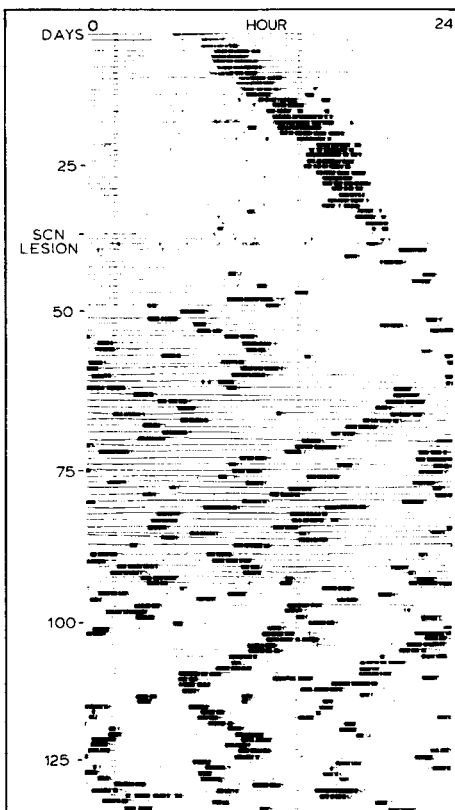
have to be made so that the concentration of nitrogen in each structure may be measured easily; the thermodynamics of each stage of the aggregation have to be found; and ^{15}N isotope shifts in the optical features of the platelets could establish more about the role of nitrogen in the platelets. Although much still has to be done, the migration of nitrogen in diamond is turning out to be an extremely profitable line of study. □

Are the suprachiasmatic nuclei the location of the biological clock in mammals?

from Fred W. Turek

OVER the past twenty-five years, largely due to the efforts of Colin Pittendrigh and Jürgen Aschoff, it has been demonstrated that the generation of circadian (that is, approximately 24-hour) rhythms in animals depends on an internal biological clock. The endogenous oscillator is normally synchronized by environmental factors, principally the photoperiod, to the 24-hour rotation of the Earth upon its axis. Hundreds of circadian rhythms have been described and the formal properties of circadian systems analysed. Recently, attention has switched to the location of the biological clock(s) and the mechanisms by which the clock is coupled to the many rhythms it drives. Hundreds of experiments in scores of laboratories around the world have been carried out in an attempt to elucidate the neural elements involved. However, it is only within the past few years that the neuroscience community has begun to apply the entire armamentarium of neural techniques to the study of circadian rhythms. These initial studies have already yielded a number of important findings and have laid the groundwork for future work involving the search for the circadian clock in animals.

In mammals, the hypothalamic suprachiasmatic nuclei (SCN) have become the centre of attention. Compelling evidence that this region of the hypothalamus plays an important part in circadian organization was first provided by Stephan and Zucker¹ and Moore and Eichler² when they discovered that destruction of the SCN in rats altered both the entrainment by light/dark cycles and generation of circadian rhythms. Since these pioneering studies, many researchers have observed that bilateral lesions of the SCN in rodents disrupt a variety of circadian rhythms including drinking, eating, locomotor behaviour, plasma corticosterone, pineal *N*-acetyltransferase activity, sleep, heart rate and body temperature. In addition, SCN lesions disrupt oestrous cyclicity and photoperiodic time measurement, two parameters which depend on the circadian system for temporal information³.



Continuous record of the wheel-running activity of a male hamster maintained in constant light. Successive 24-hr days are plotted from top to bottom. On day 37 of this record, the animal received a bilateral lesion of the SCN which resulted in a clear disruption of the normal circadian rhythm of locomotor activity (from G.E. Pickard and F.W. Turek, unpublished results).

Since it is now quite clear that multicellular organisms are made up of a population of circadian oscillators, the search for the biological clock is really the search for that clock or system of clocks which coordinates the various internal oscillators with each other and the outside environment. Evidence for the existence of a population of oscillators comes from many different experiments including some that involve the destruction of the SCN in rodents. Early studies indicated

that lesions of the SCN resulted in the complete loss of entrainment and the abolishment of free-running circadian rhythms but more extensive studies by Rusak⁴ in the golden hamster suggested that a variety of independent rhythmic oscillators exist in the absence of the SCN. The figure shows the dramatic effect that lesions of the SCN can have on the circadian rhythm of locomotor activity in the hamster. On some days (for example, days 87–96) no rhythmic pattern is obvious, while on other days bimodal (about 12 hr: for example, days 100–111) and trimodal (about 8 hr: for example, days 113–120) activity patterns are generated. Rusak postulated that such patterns indicate that circadian oscillators continue to function in these animals, but the SCN are necessary to integrate these oscillators into a single circadian framework. Further evidence that at least some circadian rhythms persist in mammals after destruction of the SCN comes from work on squirrel monkeys. Although the circadian rhythms of behavioural activity, feeding and drinking are abolished following bilateral destruction of the SCN, the rhythm of body temperature persists⁵.

Attempts have been made to locate the endogenous biological clock by determining the pathways which connect the environmental factors, principally light, to the entrained circadian system. A direct retino-hypothalamic tract (RHT) which terminates in the SCN has been observed in many mammals⁶ and it has been suggested that this is the primary pathway by which light information reaches the circadian system. However, this has been difficult to prove conclusively because the close proximity of the SCN to the optic chiasm in rodents has not made it possible to sever the RHT without destruction of the SCN. Indirect evidence of a major role for the RHT is the observation that rodents can entrain to a light/dark cycle when all known retinal inputs to the brain, except the RHT, have been destroyed. Nevertheless, disruption

of the primary optic tract, leaving the RHT intact, can alter the entrainment pattern of the locomotor activity rhythm in hamsters⁷. The relative importance of the RHT and other retinal inputs to the brain for the entrainment of the circadian system by light still remains to be determined.

Through the use of recently developed neural anatomical tracing and staining techniques, new details about the morphology and the afferent and efferent connections of the SCN have emerged⁸. The SCN is a complex nucleus that has a number of recognizable subdivisions and within each division there is a heterogeneous population of neurones. Morphological studies of the SCN suggest that there is a great deal of intercellular communication within one nucleus as well as between the nuclei⁸⁻¹⁰. Inputs to the SCN arise from the retinae, ventral lateral geniculate nuclei and midbrain raphe nuclei, and a number of other inputs may exist¹⁰. Interestingly, the number of retinal ganglion cells projecting to the SCN is quite small and certain classes of ganglion cell are more likely to project to the SCN than others¹¹. Relatively little is known about the efferent projections from the SCN but, in general, it appears they leave dorsally and caudally and many terminate among cells in various hypothalamic nuclei¹².

Direct evidence for the ability of the SCN to generate circadian rhythms has been supplied by Inouye and Kawamura¹³. They found clear evidence of circadian rhythmicity in neural activity in a variety of different brain regions when they recorded multiple unit activity with extracellular electrodes. When a Halasz knife was used to create a hypothalamic island containing the SCN, they found that circadian rhythmicity in spontaneous neural activity was abolished in all brain regions outside the island but persisted inside the island. This suggests that the SCN region is capable of generating circadian rhythms without input from elsewhere and that the rhythmicity in other brain regions is dependent upon input from the SCN. Circadian fluctuations in glucose consumption and protein synthesis^{14,15}

have also been observed in the SCN, although it is not known whether the rhythms are intrinsic to the SCN region itself.

A number of laboratories are attempting to record neural activity from the SCN in hypothalamic slices maintained *in vitro*. In a preliminary paper, Groos¹⁶ reported that single units from the rostral hypothalamus (including the SCN) maintained *in vitro* exhibited clear circadian rhythms in their firing rate and suggested that neural rhythms were intrinsic to the SCN.

Very few studies have examined the neurochemistry of circadian systems in mammals. Zatz¹⁷ has demonstrated that the infusion of carbachol, a cholinergic agonist, adjacent to the SCN could mimic the effects of light on the circadian rhythm of rat pineal *N*-acetyltransferase activity. These results suggest that acetylcholine may be involved in the photoentrainment of mammalian circadian systems and point out the potential usefulness of the neuropharmacological approach in determining the mechanisms by which

circadian rhythms are entrained and self-generated.

Most of our understanding of the role of the SCN in the generation of circadian rhythms has been derived from investigations with small rodents, particularly rats and hamsters. However, recent studies involving lesions of the SCN region in lizards, birds and primates^{5,18} indicate that this region of the brain plays a central part in the circadian organization of many, if not all, vertebrate species.

Whether the SCN represent the master biological clock, or a major component of a complex circadian system, in mammals remains an unanswered question. However, whatever the precise role of the SCN in the generation of circadian rhythms turns out to be, one thing is clear; the SCN have provided neuroscientists with a starting point for the eventual elucidation of the neural basis of circadian rhythmicity in mammals. □

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Towards the numerical magnetosphere

from Martin Brown

THE SOLAR WIND is a tenuous stream of hot protons and electrons originating in the solar corona and flowing approximately radially outwards from the Sun into the Solar System at speeds of $\sim 400 \text{ km s}^{-1}$. The Earth's dipolar magnetic field presents an obstacle to this flow and excludes it from a cavity known as the magnetosphere. Because the speed of the solar wind is greater than all hydromagnetic wave propagation speeds in the solar wind medium, the perturbing influence of the Earth on the flow reaches only to the order of ten Earth radii upstream, where a standing shock is found. Downstream from the Earth, the magnetosphere is extended in a tail to great distances.

The large quantity of data available from satellite and ground-based observations of electromagnetic fields, flow velocities and particle number densities around the Earth, both in the magnetosphere and the undisturbed solar wind, has encouraged theoretical research into magnetospheric phenomena. There is major interest in the calculation of the steady-state flow of the solar wind past the Earth and its dynamic response to changes in the solar wind. The complex geometry and the need to consider interactions between electric currents and electromagnetic fields make a complete analytical solution inaccessible, but two recent publications (Leboeuf *et al.* *Geophys. Res. Lett.* **8**; 257, 1981; Lyon *et*

al. Phys. Rev. Lett. **46**; 1038, 1981) show that techniques of numerical simulation developed for research in aerodynamics and plasma physics may be very useful. Although the application of numerical methods to the global structure and behaviour of the magnetosphere is not a new idea, previous work suffered from lack of computing power and from inadequate simulation techniques which allowed only a simplified version of the problem to be considered: the shape of the magnetosphere was assumed and a flow around it calculated (Spreiter *et al. Planet. Space Sci.* **17**; 233, 1969). Larger computers now make more ambitious models possible, and attempts to simulate the time-dependent behaviour of the magnetosphere in two and even three dimensions, treating fields and currents in a proper self-consistent way, are very fashionable.

Two-dimensional numerical models are quite common in many fields and produce respectable results, but three-dimensional simulations are still quite rare since they tax to the limit the memory size and speed of operation of the most powerful computers available. In spite of this, notable results have been achieved with three-dimensional computer codes, especially in the fields of weather prediction, controlled

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thermonuclear fusion and aircraft design. The present restriction on memory size means that rather few mesh points can be used in each coordinate direction of the finite difference models. For example, in the simulation of Leboeuf *et al.* a mesh of size $32 \times 16 \times 8$ and spacing about one Earth radius was used, allowing only poor spatial resolution. A two-dimensional simulation (Lyon *et al.*) in the plane perpendicular to the path of the Earth's orbit around the Sun gives better resolution, but since the motion of particles and magnetic field lines around the sides of the Earth is important, a distorted picture emerges without resort to *ad hoc* procedures for attempting to include these effects.

Several recognizable magnetospheric features are reproduced in these models; density maxima are found at the nose and over the polar caps, and a low-density cavity exists behind the Earth's dipole field. The cross-sectional shape of the simulated magnetosphere also has similarities with that deduced from satellite measurements. Magnetic field line reconnection, an important process by which field lines can break and rejoin to neighbouring lines through resistive current flows, occurs in both models. Reconnection takes place in the nose of the magnetosphere where the interplanetary magnetic field (IMF), arriving with the solar wind, connects with the Earth's field, and in the long tail where the magnetic field configuration, directed away from the Earth in the southern half and towards the Earth in the northern half, is maintained by a perpendicular current sheet separating these regions. Field lines from the northern and southern halves near the current sheet can break and join each other leaving a neutral line downstream from the Earth. A so-called 'open' magnetosphere results from reconnection (Dungey *Phys. Rev. Lett.* **6**; 47, 1961) since the IMF connects to the internal field of the magnetosphere, allowing entry of solar wind particles down the field lines. Although the general topology of these magnetic field line structures is reproduced in simulations, the rate of reconnection is much greater than it should be because of dissipative effects inherent in both methods which gives rise to 'numerical' diffusion of field lines. This causes the neutral line in the tail to be placed much nearer the Earth than it is in reality.

Some dynamic phenomena of interest appear in the models. Fluttering of the boundary of the simulated magnetosphere (Leboeuf *et al.*), which is also seen by spacecraft, appears to be the result of the well known Kelvin-Helmholtz instability. The two-dimensional code (Lyon *et al.*) attempts to model a geomagnetic substorm (a poorly understood, large-scale event in the magnetosphere powered by the solar wind) by switching on a southward-pointing IMF in the incoming solar wind. This causes rapid reconnection in the tail of

the model, the neutral line approaching the Earth and then receding in a way very similar to that seen in the magnetosphere and predicted by theory.

The numerical magnetosphere is, at present, a rather distant cousin of the real one, though they have many common

general features. These simulations can best be thought of as feasibility demonstrations; real advances will only come using much finer meshes on the next generation of large computers, and with the development of numerical schemes with less dissipation. □

The evolution of cooperation

from Robert M. May

MOST people are familiar with the paradox of 'The Prisoner's Dilemma', which provides an elegant metaphor for the problems of achieving mutual cooperation among members of a group. It has indeed been said (Grofman, in *Frontiers of Economics* University Publications, Virginia, 1975) that more than 2,000 papers have been written on the paradox and its philosophical implications.

In its basic form, the Prisoner's Dilemma involves two individuals, each of whom can choose either to cooperate or to defect in each of a series of discrete interactions. For these encounters, an illustrative pay-off matrix has the following character: if both players co-operate, each gains 3 units of whatever currency is at stake; if one cooperates and the other defects, the defector gains 5 units and the cooperator gets 0; if both defect, both gain 1 unit. It is apparent that, on any isolated encounter, the best strategy is to defect: if the opponent also defects, you score 1 rather than the 0 that you would have scored had you cooperated; if he cooperates, you score 5 rather than the 3 that cooperation would have gained for you. Thus, irrespective of the other player's choice, it pays to defect. But, if both players defect, both score 1 unit rather than the 3 units they could both have scored had they cooperated. Hence the dilemma.

Clearly, the players' strategic decisions will depend on the likelihood of future encounters. If the individuals know they are destined never to meet again, defection is the only rational choice. By extension, if the total number of interactions is precisely known in advance, defection seems to remain the unbeatable strategy (defection is optimal on the last encounter, and therefore also on the next-to-last, and so on, back to the first).

However, if the series of encounters is to stretch indefinitely into the future, or, more generally and more realistically, if there is always a finite probability, w , that after the current interaction the two protagonists will meet again, the strategic choices become more complex.

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Smale (*Econometrica* **48**; 1617, 1980) has recently brought the formal apparatus of the theory of dynamical systems to bear on the problem, in the limiting case when there is no finite horizon and no discounting of the future. In these models "There is always a tomorrow in our plans, and it is as important as today. . . . There is a history, a beginning of history, but no end". On the assumption that decisions may be based on some kind of summary or average of past outcomes, Smale shows that there is a class of 'good strategies' that are optimal and stable, corresponding to 'Nash equilibrium points'. These good strategies are essentially cooperative ones. Although Smale's analysis is formal and abstract, it clearly demonstrates that cooperative strategies can arise, provided some plausible general conditions are met.

A more empirical approach to the problem of finding a 'best' strategy is reported by Axelrod and Hamilton (*Science* **211**; 1390, 1980), in a paper whose title I have stolen for this *News and Views* article. In two computer tournaments, Axelrod solicited strategies for playing the Prisoner's Dilemma game (with the specific pay-off matrix defined above) from game theorists in economics, sociology, political science, mathematics, physics and evolutionary biology. The first tournament involved a total of 15 entries, which were paired against each other in a round robin tournament, with each contest lasting 200 moves. The 15 strategies varied from the very elaborate ("An example is one which on each move models the behaviour of the other player as a Markov process, and then uses a Bayesian inference to select what seems the best choice for the long run"), to one in which each choice was totally random. The winning strategy was a simple one entered by Anatol Rapoport (of the Institute for Advanced Study at Vienna). Called TIT FOR TAT, it cooperated on the first move, and thereafter did whatever its opponent did on the previous move. Axelrod's second, larger tournament had 62 entries from six countries, and the pairwise contests lasted an average of about 200 moves (specifically, there was a probability $w = 0.99654$ that any given move would not be the last). Again, Rapoport's TIT FOR

TAT emerged clearly triumphant.

In retrospect the success of TIT FOR TAT can be attributed to three features: it was never the first to defect, it immediately retaliated when provoked, and it was forgiving after just one act of retaliation.

TIT FOR TAT's emergence as the 'best' strategy from the maelstrom of contenders in Axelrod's tournaments depends explicitly on the protagonists meeting again and again. Pursuing this aspect of the problem in an analytical way, Axelrod and Hamilton show that (with the pay-off matrix defined above) TIT FOR TAT will always win provided the probability, w , for a given pair of players to meet again exceeds $2/3$. More generally, if the reward for mutual cooperation is R units, for mutual defection is P units, and for cooperation-defection is T to the defector and S to the cooperator, then the strategy of TIT FOR TAT will dominate all others provided that the probability, w , of a subsequent encounter exceeds

$$(T - R)/(R - S)$$

Notice that, in this general case, we must have $T > R > P > S$ for the Prisoner's Dilemma to exist; also we require that $R > (S + T)/2$ (otherwise alternation of cooperation and defection is superior to sustained mutual cooperation).

These heuristic and theoretical studies of the Prisoner's Dilemma are of interest in their own right. Axelrod and Hamilton, however, go further to make a convincing case that this model sheds light on the way cooperation may evolve by darwinian selection in the natural world, among groups of individuals who are not necessarily closely related.

Axelrod and Hamilton emphasize that a formal theory for the evolution of cooperation needs to answer three questions. First, how can a cooperative strategy get an initial foothold in an environment which is predominately non-cooperative? Second, what type of strategy can thrive in a varied environment composed of other individuals using a wide diversity of more or of less sophisticated strategies? Third, "under what conditions can such a strategy, once fully established, resist invasion by mutant strategies (such as 'cheating' by pure defectors)?" The studies of TIT FOR TAT answer these questions about initial viability, robustness and stability. Provided the probability that interaction between two individuals will continue is sufficiently great, cooperation based on reciprocity can indeed get started in an asocial world, can flourish in a variegated environment and can defend itself once fully established.

In sketching tentative biological applications of their ideas, Axelrod and Hamilton note that there are two main requirements for cooperation to evolve by this broad route. The first is that "an individual must not be able to get away with defecting without the other individuals being able to retaliate effectively.

The response requires that the defecting individual not be lost in an anonymous sea of others. Higher organisms avoid this problem by their well developed ability to recognize many different individuals of their species, but lower organisms must rely on mechanisms that drastically limit the number of different individuals or colonies with which they can interact effectively". The second requirement is that the probability w for the same two individuals to meet again must be sufficiently high.

One way to help keep the interactants together is to use some fixed place of meeting. Thus aquatic cleaner mutualisms (in which a small fish or a crustacean removes and eats ectoparasites from the body, or even the inside of the mouth, of a larger fish which is its potential predator) "occur in coastal and reef situations where animals live in fixed home ranges or territories"; they seem to be unknown in the free-mixing circumstances of the open sea. Pursuing this line, the authors also argue that ant colonies participate in many cooperative mutualisms, whereas honeybee colonies — which are much less permanent in place of abode — have no known cooperative symbionts but many exploitative parasites.

An important simplification in the work of Axelrod and Hamilton, and in Axelrod's computer tournaments, is that all pay-offs are counted equally. The future is not discounted. In many

biological applications, however, future gains (measured in terms of reproductive effort or other coinage related to darwinian 'fitness') may be worth much less than current gains. One offspring in the hand can literally be worth two in the future. I think Axelrod and Hamilton's analysis can be widened to include such discounting of future gains, at least in simple cases, by formally redefining the probability of future encounters, w , to include an appropriate discount factor. If this is so, it adds a further requirement that must be satisfied before cooperation can evolve: not only must there be a sufficiently high probability that any two protagonists will encounter each other again, but also the gains from such future encounters must not be too heavily discounted. This consideration is an additional restriction on the biological circumstances under which cooperation may be able to evolve along the lines envisioned by Axelrod and Hamilton.

In a survey of organisms ranging from bacteria to primates, Axelrod and Hamilton indicate many examples where aspects of territoriality, mating systems or disease may be related to the evolution of cooperation. Intriguing though these speculations are, I think the real importance of their paper is in providing a rigorous, formal example of a mechanism whereby cooperation based on reciprocity may evolve.

New twists to DNA and DNA-carcinogen interactions

from Stephen Neidle

THE discovery of left-handed DNA structures (the Z forms) has undoubtedly provided a major impetus to the re-awakening of interest both in nucleic acid structure generally and in wider questions of nucleic acid recognition. Such recognition, whether by proteins (see D.R. Davies *News and Views* 290; 736, 1981), or by smaller molecules such as drugs, mutagens and carcinogens, must to some extent be specific to the sequence of the nucleic acid residues involved. It is now apparent from the crystal structure analysis¹ of the dodecanucleotide DNA fragment d(CGCGAATTCGCG) that subtle, yet distinctive, sequence-dependent structural features occur at differing points on a classical right-handed B-DNA-type double-helical nucleic acid. These are seen

in an extreme form in an alternating dG-dC sequence which has the potential of existing in the very different Z-DNA left-handed form. It is thus in principle interesting to ask whether such aberrant regions of structure are involved in recognition processes.

A central question concerns the co-existence of left- and right-handed helices within the same trait of DNA. Recently, it has been reported that oligomeric (dG-dC) fragments of known size have been successfully inserted into DNA restriction fragments². Both circular dichroism and phosphorus (³¹P) NMR measurements showed that these alternating sequences behaved in a manner typical of the B→Z transition induced by increases in salt concentration. Since the non-alternating sequences in the fragments studied did not show such behaviour, it is clear that both forms of DNA can co-exist, with a roughly eleven-base pair interface between them. The visualisation of this intermediate area

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will surely provide fertile ground for the model-builders, at least until relevant crystal structures are obtained. Even though the result implies that a considerable length of (dG-dC) oligomer is required for full Z character to be shown in between these interfacial regions, it is not unreasonable to suppose that rather shorter (dG-dC) traits will have some abnormal structural character.

The salt-induced conformational transition of DNA (B→Z) is the basis of all experimental probes of left-handed DNA in solution. However, it remains true that until recently, it has been no more than a strong presumption (albeit a logical one) that the high-salt form in solution does indeed correspond to the left-handed structures observed in the crystalline solid state. Evidence is now accumulating that this assumption is a valid one. Thus, Sarma and his associates³ have shown that the observed proton NMR chemical shift data for high-salt poly(dG-dC). poly(dG-dC) corresponds very well with the spectral data calculated theoretically from a Z-DNA helical structure, and not at all with that from B-DNA. Furthermore, it is evident that this close agreement almost certainly excludes other more recherché DNA structures, at least in this context. Interesting structural questions have been posed by the observation⁴ that the alternating copolymer, when fully methylated at the cytosine 5 position, undergoes a B→Z transition at a roughly physiological salt concentration which is much less than that required for the transition in the unmethylated polymer. Since it has often been suggested that C⁵ methylation in eukaryotes is related to control of gene expression, it is indeed tempting to speculate that here, at last, may be a role for Z-DNA. Indeed one keenly awaits firm evidence that any biological function actually does involve a switch of DNA helicity from B→Z. The recent production of antibodies specific for the Z form⁵ may well prove to be a powerful tool for providing such data.

It was apparent soon after the discovery of Z-DNA that certain atoms, such as the C-8 of guanine residues, are rather more exposed than in B-DNA⁶. The C-8 site is favoured in binding of the tricyclic aromatic hydrocarbon derivative acetylaminofluorene (AFF), a much-studied hepatic carcinogen. In view of this, and the knowledge that at least some mutational hot-spots (for example in

'Ames-test' histidine genes of *Salmonella* strains) contain clusters of alternating (dG-dC) residues, there has recently been much interest in the possibility that Z-DNA is involved in carcinogenic processes.

Interaction of poly(dG-dC). poly(dG-dC) with the active species *N*-acetoxy-AAF results in random modification⁷⁻⁹ of the polynucleotide, which then shows Z-like character even at low salt concentrations. No helix disruption seems to have occurred as the polynucleotide is not susceptible to S₁ nuclease which attacks single-stranded regions. The physical model that emerges is of AAF attaching to guanine residues

which then for purely steric reasons have to be in the *syn* conformation of Z-DNA itself. On the other hand, with non-alternating (essentially random) sequences AAF binding involves some local destabilisation of the double helix, such as is suggested by a base displacement model⁹. This has the *syn* guanine residue no longer paired but swung out and displaced from the double helix. One suspects that these two types of AAF-binding models are actually closely related in structural terms. It will be of some interest to see how they differ biologically, such as in their susceptibility to excision repair. □

Diphtheria toxin: which route into the cell?

from Simon van Heyningen

A single molecule of diphtheria toxin can kill a eukaryotic cell. Several other bacterial protein toxins also have comparable activity and all are large molecules which have to penetrate the cell membrane before they can act. Diphtheria toxin was the first of these to be understood, at least in outline, but several important details have only recently been published.

Diphtheria toxin (molecular weight 60,000) is made up of two polypeptide chains, fragment A (21,000) and fragment B (39,000), linked by a disulphide bond. The toxin first binds to cells through the B fragment. This leads to the entry into the cell of fragment A — which then folds to an active conformation, and catalyses a reaction in which the ADP-ribose of NAD is transferred to elongating factor 2 (EF2), an essential protein component of ribosomal protein synthesis. Since ADP-ribosylated EF2 is inactive, protein synthesis stops and the cell dies. It is because fragment A is an enzyme that the toxin is so active.

Fragment A is highly specific. Its only significant substrate is a single amino acid residue in EF2 whose structure has recently been determined by NMR¹. It is a complex derivative of histidine named 'diphthamide', 2-(3-carboxyamido-3-[trimethylammonio] propyl)histidine. The specificity of the toxin is not, however, just for diphthamide, but for something more complicated: peptides containing diphthamide isolated from tryptic digests of EF2 are not ADP-ribosylated.

Since the toxin ADP-ribosylates the EF2 of all eukaryotes (and even of the archaeobacteria), diphthamide must be highly conserved. The amino acid sequence around it also shows little species variation. It is hard to believe that so complicated a

modification has evolved to provide a substrate for diphtheria toxin, so one might expect diphthamide to be required for the action of EF2. Surprisingly, however, mutants that lack diphthamide (and are insensitive to toxin) show no defect in protein synthesis. These mutants can be divided into three complementation groups, in agreement with the number of enzymic modifications which the structure of diphthamide suggests would be needed to make it from histidine. Diphthamide is presumably also the substrate for ADP-ribosylation by the toxin of *Pseudomonas aeruginosa*, which works in a very similar way, but not for cholera or *E. coli* toxins which are less specific and ADP-ribosylate different proteins.

Before fragment A can have its effect on EF2 it first has to pass into the cell. Entry begins with the binding of fragment B to a receptor on the outside of the cell membrane. The nature of this receptor was unknown until recently (although it had been mapped on the human chromosome), but it has now been identified as a glycoprotein that can be isolated from guinea-pig cells by immunoprecipitation². It is not a true receptor in the endocrinological sense, since binding to it does not in itself have any effect. Rather, it is a prerequisite for entry that the toxin binds firmly to the cell. Quite different ways of doing this are effective; derivatives of fragment A with other cell-surface ligands such as antibodies or even subunit B of cholera toxin can still enter the cell.

Once the toxin is bound there are two different ways in which the A fragment could enter the cell. It could cross the

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membrane directly (either by itself or with a carrier), or it could be endocytosed, in which case the problem of crossing the membrane into the cytosol would still remain. For this and other reasons, endocytosis has not been a favoured hypothesis, but some new experiments have made it seem much more likely³.

Uptake of toxin is greatly increased at low pH. When cells are exposed to toxin at pH 4.5 for ten minutes, the rate of decrease in protein synthesis is the same as it would have been using a thousand times as much toxin at neutral pH. As the pH is low in the lysosomes, the results immediately suggest that that is where toxin might normally enter the cell. There is other evidence that this is what happens. When the pH inside the lysosomes is increased by incubating the cells with ammonium chloride, the toxin is very much less active (although its binding to the cell surface is not decreased). However, this protective effect of ammonium chloride is abolished if the cells are exposed to low pH for even as little as 20 seconds. Experiments using antisera suggest that, at neutral pH, toxin is trapped in intracellular vesicles, but, at low pH, it can penetrate the membrane directly; lysosomes are no longer involved and so ammonium chloride has no effect.

What may normally happen is that the toxin is taken up by endocytosis into intracellular vesicles which then fuse with lysosomes. Most of the toxin will be destroyed by the lysosomal enzymes, but at the low pH in the lysosomes, a few molecules of fragment A will escape into the cytoplasm.

How does fragment A penetrate membranes at low pH? Recent experiments using artificial lipid bilayers may provide a clue⁴. Diphtheria toxin and a mutant (crm 45) that lacks the receptor-binding region of fragment B can bind to these layers and form transmembrane channels through them. These channels are formed best when one side of the membrane is at low pH and the other is neutral, and their width (perhaps about 18 Å diameter though there is some disagreement) is probably large enough for fragment A to pass through provided it unfolds to a fully extended form. Fragment A is a very stable protein which could easily refold to a native conformation afterwards.

The formation of these channels probably involves more than one molecule of fragment B transversing the membrane. Fragment B is well able to do this: it has a

hydrophobic domain that could dissolve in the core of the membrane lipids, and a hydrophilic domain that could interact with the polar head groups⁵.

It seems unlikely that the mechanisms used by bacterial toxins for entering cells

and affecting their metabolism are unique. Rather, they may also be used in other, as yet unidentified, ways. Study of these curious proteins should help the understanding of the normal properties of cells.

The actin-myosin interface

from Howard White

UNDERSTANDING the molecular mechanism of muscle contraction will almost certainly require a detailed knowledge of the structure of, and the interaction between, the primary contractile proteins, actin and myosin. A simple model in which each myosin-S1 binds to a site completely contained with a single actin subunit has been generally assumed from the binding stoichiometry of one myosin-S1 per actin subunit. The observation made several years ago by Chantler and Gratzer (*Biochemistry*, **15**, 2219; 1976) that myosin-S1 binds to monomeric actin covalently bound to sepharose strengthened the case for a simple one-to-one interaction between each myosin head and actin filament subunit.

In this issue of *Nature* (p.301) Mornet *et al.* report that a water soluble carbodiimide crosslinks the 95K dalton heavy chain of myosin-S1 to two actin subunits and conclude, that the binding site for each myosin-S1 to filamentous actin spans two actin subunits or is at least close enough to form zero-length crosslinks with two actin subunits. Although crosslinking experiments of this type have a rather perilous history, the authors have devised an extensive series of controls that considerably strengthen their argument.

First, the crosslinking reaction is quite specific. Actin activated with carbodiimide is crosslinked to myosin-S1 but myosin-S1 activated with carbodiimide is not crosslinked to actin. The crosslinking reaction is prevented by either pyrophosphate or MgATP, which specifically dissociates myosin-S1 from actin. Second, the composition of the crosslinked species, two actin subunits and one myosin-S1 heavy chain, initially suggested by a molecular weight of 180K dalton on SDS gels was verified by a molar ratio of two³H-actin to ¹⁴C-S1 in the crosslinked polypeptide. Third, the possibility that S1 binding to actin enabled intermolecular crosslinks to be formed between actin subunits was eliminated by the following experiments. Trypsin specifically cleaves the heavy chain of myosin-S1 bound to f-actin into two peptides of 75K and 22K daltons; cleavage of carbodiimide crosslinked actomyosin-S1 produces two peptides with molecular weight of 120K and 64K daltons. The proteolytic fragments of the

crosslinked actomyosin-S1 were identified as 22K (S1)-actin and 75K (S1)-actin by specific fluorescent labeling of the actin and myosin-S1 before crosslinking and cleavage.

Hence, two different regions of the myosin-S1 heavy chain appear to be cross-linked to separate actin subunits. The authors propose a model in which the myosin-S1 binds to a site comprising two actin subunits on opposite strands of the actin helix. However, their data and recent three-dimensional reconstructions obtained from electron micrographs of negatively stained actomyosin-S1 filaments (Taylor and Amos *J. molec. Biol.* **147**, 297; 1981) are equally compatible with the myosin-S1 being bound to two actin subunits on the same strand of the actin filament.

The enzymatic properties of the crosslinked actomyosin may provide a new tool to help unravel the increasingly complex mechanism of actomyosin ATP hydrolysis. Crosslinked actomyosin-S1 hydrolyzes MgATP at a rate comparable to the rate of hydrolysis by actomyosin-S1 in solution extrapolated to infinite actin concentration. There is, however, one notable difference — the large dependence of rate upon ionic strength that is so characteristic of actomyosin-S1 in solution (primarily due to the ionic strength dependence of the equilibrium of $M \cdot ADP \cdot P_i + \text{actin} \rightleftharpoons \text{acto} \cdot M \cdot ADP \cdot P_i$, which precedes the rate-limiting step) is absent. Crosslinked actomyosin-S1 thus appears to provide a covalent model for the so-called non-dissociating pathway of actomyosin ATP hydrolysis that could previously be observed only at a very low, non-physiological ionic strengths.

One may well ask how all of this fits the notion of rotation of the myosin heads bound to actin — the postulated work-producing step of most of the popular models of contraction today. The molecular basis of muscle contraction is indeed far from being solved and continues to provide both the structuralists and biochemists with more riddles to decipher.

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ARTICLES

Magma chamber profiles from the Bay of Islands ophiolite complex

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Models of the oceanic lithosphere derived from constraints imposed by the geology of ophiolite complexes require a magma chamber to produce the cumulate and other plutonic portions of the oceanic crust and upper mantle. Evidence from the Bay of Islands ophiolite complex indicates that layered plutonic rocks have not formed solely due to differential crystal settling to the horizontal floor of a magma chamber, but have formed largely by heterogeneous nucleation and in situ crystallization along gently inclined as well as steeply inclined bounding surfaces of a large magma reservoir. Coherent, well defined and systematically oriented, large-scale, arcuate patterns described by mesoscopic igneous layering have been identified in the plutonic sections of two ophiolite massifs in the Bay of Islands complex. These layering patterns seem to outline the shape of a large, continuously evolving, steady-state magma chamber that extended along a ridge axis and ended at a transform fault intersection.

EVIDENCE from marine geology, geophysics and theoretical considerations suggests that steady-state magma chambers may exist beneath fast-spreading accreting plate boundaries¹⁻⁶ while only small ephemeral chambers may occur along slow-spreading plate boundaries⁷. Previous models for the evolution of the oceanic crust and upper mantle have been built around the concept of a steady-state chamber and generalized observations from ophiolite complexes^{8,9-11}. Ophiolite complexes provide cross-sectional views of large-scale, metamorphic-plutonic-volcanic layered sequences widely believed to be on-land exposures of ancient oceanic lithosphere^{11,12}. There is general agreement that volcanic rocks and underlying sheeted diabase dykes are formed by continuous crustal extension and magmatism at an oceanic spreading centre. The evolution of the underlying stratiform plutonic assemblage, however, has been modelled in several different ways. All models must deal with the problem of producing a subhorizontally layered assemblage from a continuously widening subvertical crack. All previous steady-state models assume the formation of cumulates occurs only along subhorizontal surfaces, but this requires extremely large magma chambers^{9,11} unless tectonic rotations occur⁸. Most interpretations of ophiolites suggest that cumulate layering should be restored to a subhorizontal orientation parallel to the large-scale stratiform structure of the complex^{11,13,14}. Evidence from the plutonic sections of the Bay of Islands ophiolite complex, however, indicates that 'cumulates' have formed along steeply inclined magma chamber walls and that the final geometry of the fine-scale igneous layering is much more complex than that assumed in most previous models or than is apparent from reconstructions of dismembered ophiolites.

Bay of Islands ophiolite complex

The Bay of Islands ophiolite complex (Fig. 1) is located along the west coast of the island of Newfoundland, Canada. The ophiolite is exposed in four large massifs (each ~20 km across) which are considered to be the dissected remnants of a once continuous allochthonous sheet of early Ordovician oceanic crust and upper mantle. The tectonic setting and rationale for the interpretation of the complex as oceanic lithosphere are discussed elsewhere¹⁵⁻²⁰. The large-scale stratiform structure of the ophiolite complex (Fig. 2) includes (from base to top): metamorphic tectonic periodotite, ultramafic and mafic plutonic rocks,

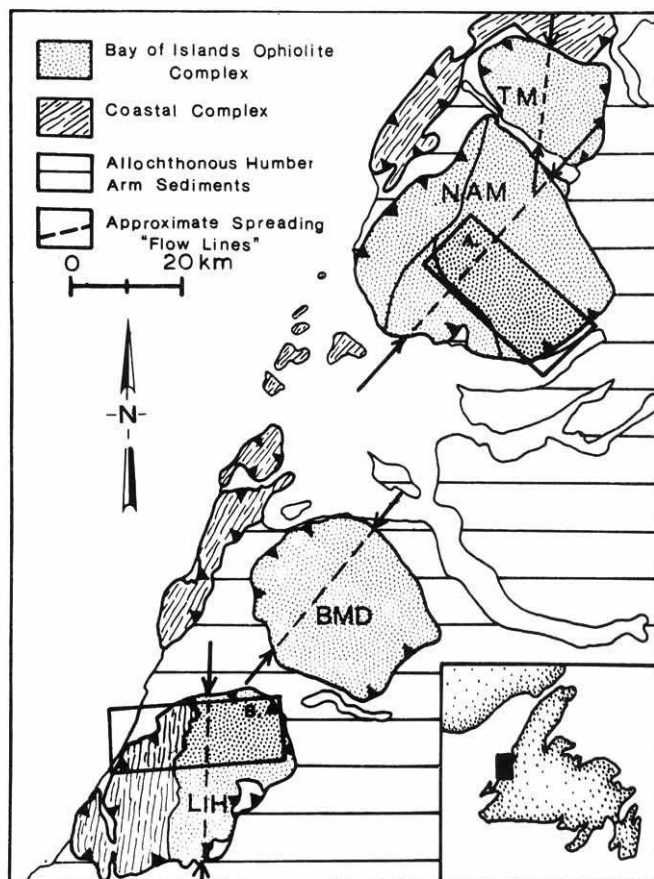


Fig. 1 Generalized geological setting of the Bay of Islands ophiolite complex. Inset shows location in western Newfoundland. Boxes *a* and *b* show location of sketch maps in Fig. 3. Massifs: TM, Table Mountain; NAM, North American Mountain; BMD, Blow-Me-Down Mountain; and LH, Lewis Hills. Arrows and dashed lines indicate trace of inferred seafloor spreading flow lines.

sheeted diabase dykes, and basaltic volcanic rocks. The Bay of Islands ophiolite clearly conforms to the current working definition of an ophiolite complex¹¹ and includes wide outcrop areas suitable for detailed studies. Although the uppermost units have in some places been removed by erosion, and some

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sections are structurally dismembered, uninterrupted sections interpreted as exposures of former upper mantle to sea floor, are well preserved.

The Bay of Islands complex may be divided into two petrogenetically distinct components (Fig. 2), a residual upper mantle assemblage depleted by subcrustal partial fusion and extraction of basaltic liquids^{8,21,22}, and a 'magmatic' component, the assemblage derived by differentiation of those basaltic extracts. The magmatic component can be divided into two large-scale structural assemblages²³. One is an upper intrusive-extrusive carapace of mafic volcanics and sheeted diabase dykes interpreted as the continuously extended and rapidly cooled lid of a magma chamber. The second is a plutonic group believed to have formed by fractionation of basaltic melt in a crustal-level magma chamber. This group includes (from base to top): (1) layered ultramafic cumulates, mainly dunite, wehrlite, clinopyroxenite and chromitite; (2) interlayered ultramafic and gabbroic cumulates (the transition zone), mainly dunite, wehrlite, troctolite, olivine-gabbro and anorthosite; (3) layered gabbroic cumulates, mainly troctolite, olivine-gabbro and gabbro; and (4) isotropic gabbroic rocks, mainly gabbro and metagabbro. This type of plutonic association has been observed in many ophiolite complexes and, by analogy to continental stratiform intrusive bodies, is interpreted in terms of magma chamber processes^{11,24}. We believe that the internal structure of the plutonic group of the magmatic component can provide some useful constraints on the dimensions and shape of the magma chamber(s) from which the complex formed.

It is assumed in reconstructing the massifs, which are now folded on a large scale, that major, lithostratigraphic-unit contacts had a subhorizontal orientation before obduction and that the mean orientation of diabase dykes was parallel or subparallel with the ridge axial plane at the time of formation. In general, two distinct types of crustal cross-sections are preserved in surface exposures of the complex. One type is reconstructed

as an approximately vertical section through the Bay of Islands complex and is oriented parallel to a spreading flow line (that is, normal to the palaeo-spreading centre and parallel to fracture zones). This type of section is exposed in map view of the northern three massifs (Figs 1 and 3a) due to tilting of major lithostratigraphic units about obduction-related, synclinal fold axes oriented approximately parallel to the spreading direction²⁵. The second type of section is preserved in surface exposures of the southernmost massif, the Lewis Hills (Figs 1 and 3b). The Lewis Hills section exposes the intersection of the Bay of Islands complex with a subvertical zone containing a highly deformed and metamorphosed ophiolitic assemblage, the coastal complex, which is interpreted as oceanic crust and upper mantle with a history of transform fault tectonics predating its juxtaposition with the Bay of Islands complex^{20,26}. The outcrop pattern of the Lewis Hills massif is controlled by a large scale, obduction-related syncline; its axis plunging gently to the north-northwest approximately parallel with the inferred spreading direction and the trend of the coastal complex. The western part of the massif lies in the core of this syncline and exposes the subvertical, highly-deformed coastal complex. The eastern side of the massif is tilted to the west and exposes the deeper structural levels of the plutonic section and the upper levels of the harzburgite section of the Bay of Islands complex.

Internal structure of the plutonic group

Contrary to assumptions commonly made in the reconstruction of dismembered ophiolite sections, the mesoscopic-scale cumulate layering is generally not parallel to the contacts between the large-scale lithologic units as first noted by Dewey and Kidd⁸. Broad arcuate patterns, defined by cumulate layering oriented up to 90° oblique to lithologic unit contacts, are observed within the plutonic group. Although in detail these arcuate patterns

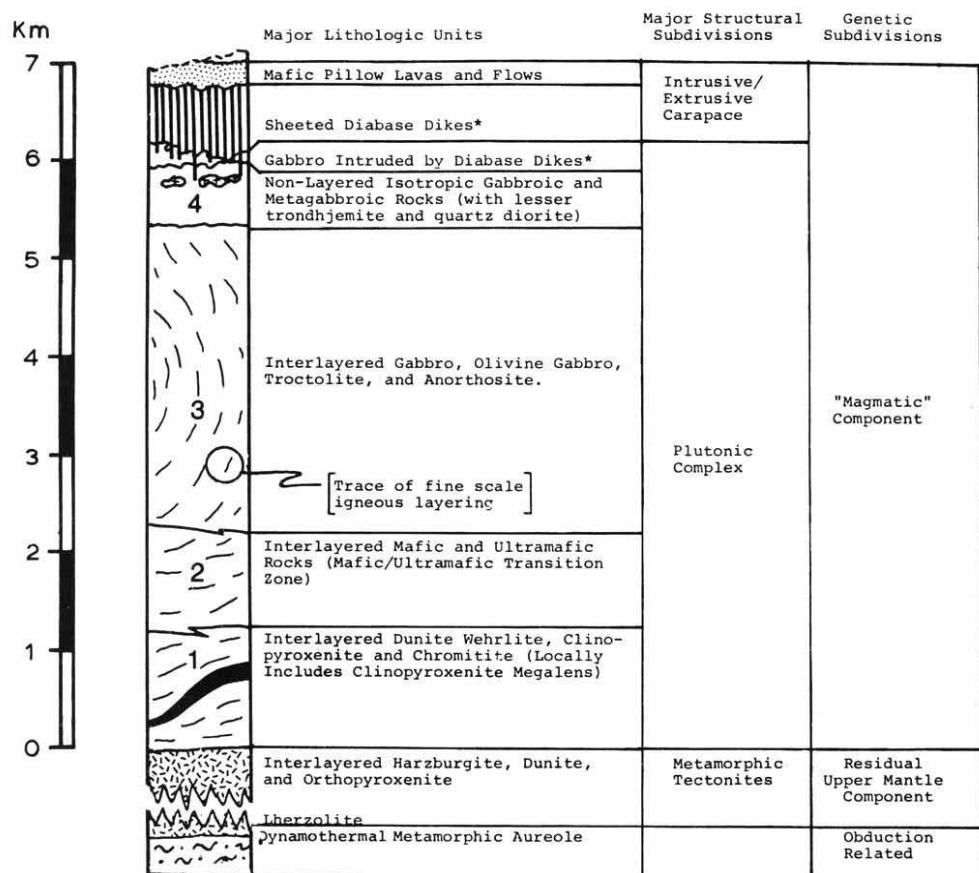


Fig. 2 Generalized columnar section through the Bay of Islands ophiolite complex and interpretations of major lithologic units. The magmatic component is to scale; the residual component is condensed (actual thickness approaches 4–5 km).

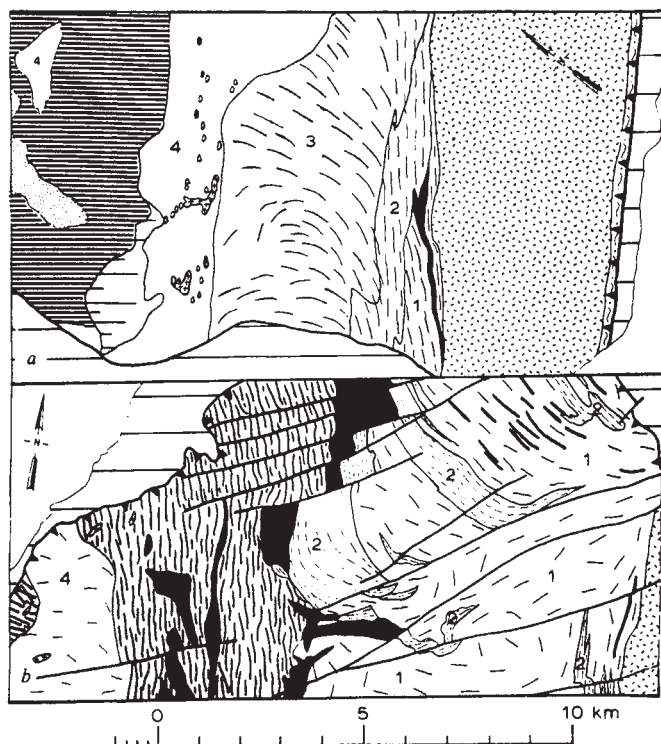


Fig. 3 Geological sketch maps of two areas in the Bay of Islands ophiolite complex (see Fig. 1). *a*, Southern half of the North Arm Mountain massif. Shading as in Figs 1 and 2, ruled lines on the diabase dyke units are oriented in the approximate direction of the mean dyke trend. *b*, Central part of the Lewis Hills massif. Light grey shading and bold squiggles indicates highly deformed rocks of the coastal complex including highly strained ultramafics, mertagabbros, amphibolites, and granulites; dark grey shading indicates intrusive periodotite bodies of the coastal complex; bold dashed lines are chromitite megalens units. Both maps are oriented with the mean trend of diabase dykes (the palaeo-ridge axis trend) parallel with the longer edges of each map.

appear to be quite irregular, the large-scale relationships are clear. In two of the massifs, North Arm Mountain and the Lewis Hills (Fig. 3), detailed mapping has revealed especially well-defined arcuate structures described by fine-scale igneous layering. The patterns identified in these two massifs, however, have very different geometric relationships with respect to the inferred spreading direction during formation of the complex. (The textural terminology and classifications systems developed for rocks believed to have originated by crystal settling^{27,28} can be applied to many coarse-grained plutonic rocks regardless of origin. We do not interpret layered plutonic rocks of the Bay of Islands ophiolite as cumulates in the genetic sense, but use the terms cumulate, cumulus, primocryst, intercumulus and post-cumulus because they are descriptive regardless of the rocks origin and because a satisfactory, non-genetic terminology is not well developed. For further discussions see refs 29, 30.)

The southern half of the North Arm Mountain massif²⁵ (Fig. 3a) exhibits the best defined example of this arcuate structure in the Bay of Islands complex. We emphasize, however, that similar discordant relationships between cumulate layering and unit contacts have been mapped in all of the other massifs. In the North Arm Mountain massif a nearly complete ophiolite sequence is preserved. The present-day erosional surface which truncates the sequence is nearly parallel to the inferred seafloor spreading flow lines, that is, normal to the mean trend of diabase dykes in the sheeted dyke unit. Fine-scale igneous (cumulate) layering attitudes within the plutonic group change gradually and continuously upward from subparallel to the major lithologic unit contacts (that is palaeohorizontal planes) in the basal ultramafic and transition zone units to orientations highly oblique to these contacts in the gabbroic units. At the upper

contact of the layered gabbroic rocks (Unit 3, Fig. 3a), layering attitudes are $\sim 90^\circ$ or even apparently overturned with respect to the major lithologic unit contacts. In a reconstructed section the discordant fine-scale igneous layering consistently strikes subparallel to the sheeted dyke direction while defining broad arcuate patterns as depicted in Figs 3 and 4. The axis of this arc or bend in the layering lies approximately in the plane of the major lithologic unit contacts and also in the plane of the mean dyke orientations indicating that, as exposed, the pattern essentially defines a cross-section of a half cylinder whose long axis originally lay in a subhorizontal orientation within the ridge axial plane. The convex side of the arc points towards the spreading direction which is inferred from one-way chilling statistics from the sheeted dyke unit³¹ and the sense of shear deduced along the coastal complex transform^{20,26}.

A single clinopyroxenite-rich 'megalens' paralleling fine-scale layering occurs within the ultramafic cumulates on North Arm Mountain. This megalens varies in thickness from 0 to 300 m (Fig. 3a) and consists of aggregates of small lenses or layers of dominantly clinopyroxenite, with lesser amounts of inter-layered dunite, wehrlite and chromitite. This megalens can be traced laterally for over 4.0 km as it cuts obliquely across the ultramafic cumulate section. Where the megalens intersects the lower contact of the transition zone, clinopyroxenites become interlayered with gabbroic rocks. These gabbroic layers increase in abundance with height in the transition zone at the expense of clinopyroxenite layers until the megalens cannot be distinguished.

An important relationship within the plutonic group concerns the relative orientations of fine-scale igneous layering, megalenses, the contacts between major lithologic subdivisions of the plutonic assemblage, and the direction of cryptic chemical variations. In continental stratiform intrusions, to a first approximation, fine-scale igneous layering is controlled by and lies parallel to the various major rock units. It also remains orthogonal to the direction of cryptic chemical variations. Fine-scale layering and megalenses in the Bay of Islands complex plutonic group, however, consistently maintain an oblique relationship to the contacts between the major lithologically defined subdivisions of the plutonic group and in some cases have trends parallel with the direction of cryptic chemical variations²².

Similar, but as yet less well-defined arcuate structures occur elsewhere in the Bay of Islands complex on the Blow-Me-Down Mountain and Table Mountain massifs where highly oblique layering relationships are also present. In the Lewis Hills massif^{26,32}, a different, but well-defined arcuate structure is present (Fig. 3b). Cumulate layering and (layered cyclic) megalens units up to 1.0 km thick are mutually parallel throughout the plutonic group. In the eastern half of the massif these features lie subparallel to the basal contact of the magmatic suite along that contact and in the central part of the massif layering is subhorizontal. To the west, however, fine-scale igneous layers vary continuously in orientation through approximately 90° approaching subvertical adjacent to the coastal complex. These layers commonly include local concentrations of angular detached blocks of layered gabbro. This geometry and these intrusive relationships suggests lapping of the plutonic group against the subvertical contact with high-grade metamorphic rocks of the coastal complex. The structure preserved in the Lewis Hills massif probably formed as relatively young crust of the Bay of Islands complex was accreted to older deformed crust of the coastal complex at a ridge/transform intersection^{20,26}. The axis of the large-scale arcuate structure defined in this area lies parallel to the inferred trend of the fracture zone and normal to the attitudes of undeformed diabase dykes in the massif (that is, approximately perpendicular to the axis defined for the northern massifs).

Interpretation of the internal structure of the plutonic group

Although the large-scale lithologic unit thicknesses and details of the internal structure of the plutonic group vary considerably

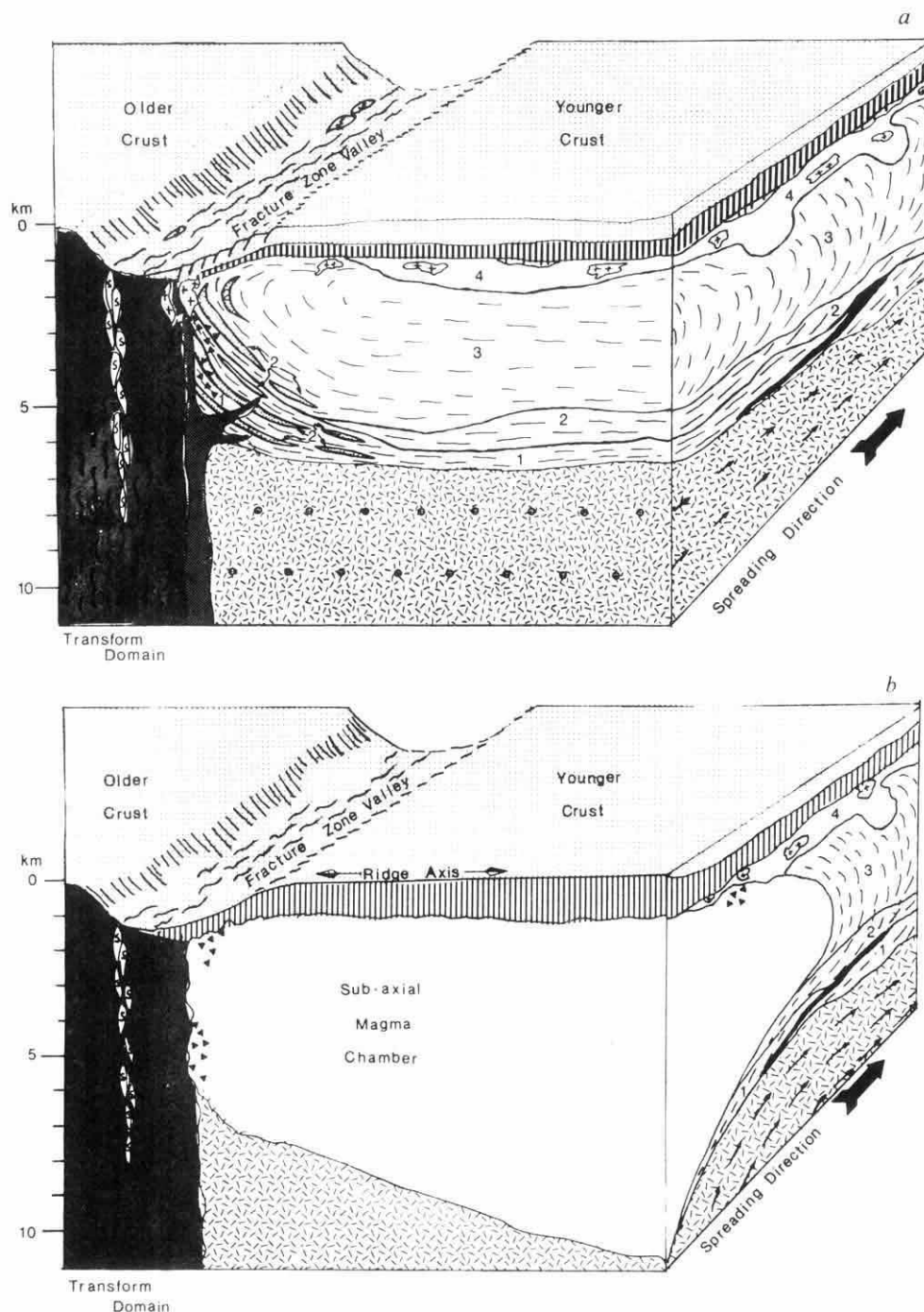


Fig. 4 *a*, Block diagram summarizing the reconstructed pre-obduction, post-accretion geology and geometrical relationships within the Bay of Islands and coastal complexes. The front face of the block diagram is oriented perpendicular to the spreading direction and crosses an inactive transform domain (the coastal complex). The other face of the diagram is oriented parallel to the spreading direction. *b*, Block diagram depicting a schematic model for the formation of the geology depicted in *a*. Orientation of diagram is similar, except that the front face is coplanar with the ridge axial plane and it crosses the transform domain at the point at which it becomes inactive. The arcuate geometry of the fine-scale igneous layering and megalenses is the product of heterogeneous nucleation, *in situ* crystallization and layer formation along the bounding surfaces of the steady-state magma chamber. Shading as in Figs. 1–3. S, serpentinite; ▲, xenoliths; small arrows, flow paths in the upper mantle.

across the Bay of Islands complex²³, these differences seem to represent variations on a fairly simple theme. In the two types of arcuate structures described above, mesoscopic and sometimes even larger-scale cumulate layering are clearly not parallel to the contacts between the large-scale lithologic units. By analogy with the inferred horizontally-layered seismic structure of contemporary oceanic crust these contacts probably originated in a subhorizontal orientation. Whereas the deepest structural levels of the Bay of Islands plutonic group show signs of extensive subsolidus deformation, we emphasize that the bulk of this group has not been penetratively deformed^{23,25,26}. Therefore, we believe that the first-order geometry of layering as described above must be the result of igneous processes because we have accounted for all obduction or post-obduction structures in the reconstructions.

Numerous studies of igneous plutons with cumulate layering indicate that cumulate textures and layering commonly can form along subvertical walls of magma chambers as well as sub-

horizontal floors^{29,30,33–36}. Integrated field and theoretical studies of cumulate rocks suggest that crystals nucleate and grow mainly along the margins of large magma chambers rather than homogeneously throughout the magma followed by vertical settling²⁹. Thus, cumulate layering may mimic the shape of the walls of such magma chambers as, we believe, is the case in the Bay of Islands complex. Any model for the origin of the igneous layering requires formation along the magma/wall (or floor) interface and therefore these layers must define isochrons. The fact these igneous layers as well as megalens units generally cut across major lithologic unit contacts and sometimes parallel the general direction of cryptic chemical variation within the plutonic group of the Bay of Islands complex implies prolonged and probably systematic physical and/or chemical heterogeneities along the bounding surface of the magma chamber.

Certain detailed aspects of the plutonic rocks, which are pertinent to the discussion of the origin of the fine-scale igneous layering in the Bay of Islands complex, also support the conten-

tion made above on the grounds of layer discordancy that much of the plutonic section formed by *in situ* crystallization processes at the bounding surface of the magma chamber. Fine-scale igneous layering in the plutonic section generally ranges in thickness from less than a centimetre to a few metres. Two types of layering are dominant. The first type, designated 'uniform layering', is characterized by uniform modal proportions and physical and textural properties. The second type, designated 'stratified layering', is characterized by size and/or modal stratification. The most common examples of stratified layers in the complex are those exhibiting modal stratification, but lacking size stratification. Size-stratified layers are present only locally and layers exhibiting both size and modal stratification are rare. Many of the stratified layers exhibit reversed stratification and some single layers exhibit both normal and reversed stratification. Where studied in detail²⁵, however, most layers (>80%) in the Bay of Islands plutonic sections are uniform in character. Consequently, the dominant sequence of layer types consists of successive uniform layers with a general lack or total absence of interlayered stratified layers. These sequences of solely uniform fine-scale igneous layers cannot be explained by the 'intermittent current hypothesis' proposed by Wager and others^{27,37} which is a model of layer formation by differential crystal settling in conditions of continuous crystallization within a magma chamber, because such a model demands that layer sequences be characterized by either alternating uniform and stratified layers, or successive stratified layers. Because of the abundance of sequences exhibiting successive uniform layers without intervening stratified layers, we believe the crystal settling mechanism of layer differentiation proposed by these workers was probably not important in the Bay of Islands complex. Goode³⁸ proposed an alternative mechanism involving differential crystal settling, not in conditions of continuous crystal nucleation, but in conditions of discontinuous nucleation. This model can, in theory, explain the existence of reversely stratified layers and can account for successively repeated uniform layers having a single cumulus (that is, primocryst) phase. Successively repeated uniform layers having more than one primocryst phase are, however, abundant in the transition zone and layered gabbroic units of the complex and they are often bounded by sharp form contacts²⁸ which involve no change in mineralogy, but simple changes in grain size, texture, or modal proportions. This type of layer sequence is not predicted or readily explained by this alternative model of layer development by differential crystal settling. We suggest that the sequences of layer types observed in the plutonic section of the Bay of Islands may be more appropriately interpreted in terms of *in situ* crystallization models rather than differential crystal settling models.

Comb layering^{34,39}, inch-scale layering⁴⁰ and intercumulus layering³⁸ are locally abundant in the plutonic section and constitute layer types which are interpreted to form by *in situ* crystallization processes^{28,29,36,38}. Colloform growth structures similar to those described within the marginal border group of the Skaergaard intrusion^{28,36} and layers exhibiting discontinuous protrusions which resemble reef-like growth structures and commonly contain elongate dendritic crystals oriented perpendicular to the regional strike of more normal layering are also commonly observed and can be interpreted in a similar fashion. Chromitites exhibiting chain textures occur in the ultramafic unit of the plutonic section and may imply *in situ* crystallization and/or heterogeneous nucleation²⁹. Oikocrystic textures characteristic of some wehrlitic and feldspathic dunites and some gabbroic rocks are difficult to interpret as the result of crystal settling and accumulation processes followed by *in situ* post-cumulus oikocrystic growth²⁵, rather, the lack of a self-supporting crystal framework of supposed, smaller primocryst phases included within the larger, supposed post-cumulus oikocrysts may indicate a wholly *in situ* origin for these rocks. Considered with evidence on the discordancy of the fine-scale igneous layering, we interpret these data to indicate that the bulk of the plutonic section has formed as the result of nuclea-

tion and growth of crystals at the bounding surface of the magma chamber. Heterogeneous nucleation of crystals at the margins of the chamber may, in part, be the result of the very low degrees of super-cooling expected in the interiors of extremely large magma chambers at mid-ocean ridges.

Lateral continuity to thickness ratios of fine-scale layers are very small in comparison to most continental layered mafic intrusion^{28,40} and even the thickest layers can seldom be followed for more than 50 m along strike. While lithologically distinct megalens units within the plutonic sections can be followed for significant distances along strike (as much as 4 km), their constituent fine-scale layers are also lens-like pinching out in either direction along strike over as much as 50 m or as little as 1 m. If these layered plutonic rocks form due to *in situ* crystallization, the lack of lateral continuity must be interpreted as the result of fairly significant lateral changes in physico-chemical conditions on a scale approximated by the lateral continuity of the layering. This may indicate that the edges of the magma chamber were periodically or continuously characterized by a fairly stagnant boundary layer³⁶ in which diffusion-controlled differentiation processes might be operative.

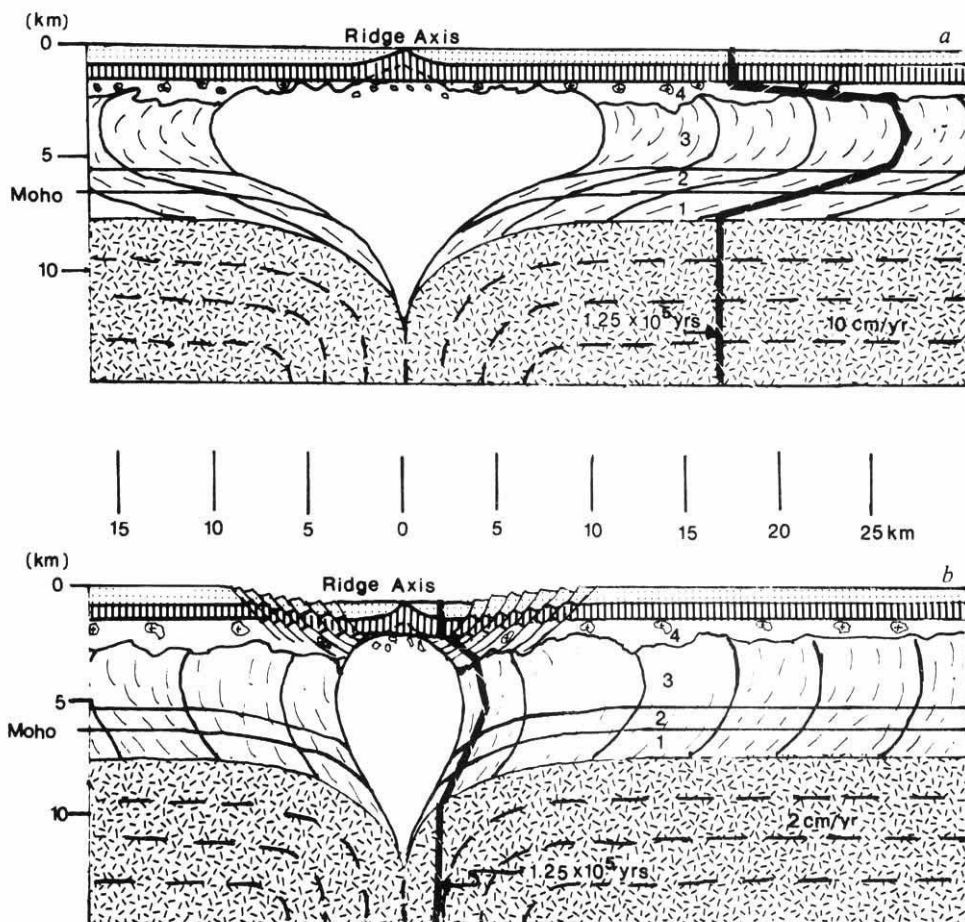
In the northern three massifs the arcuate shapes defined by cumulate layering are interpreted as profiles generated successively along one side of an elongate cylindrical magma chamber. We prefer to interpret this structure as the product of progressive growth of the plutonic assemblage as it rode first upwards and then laterally away from the spreading centre axis above a depleted-upper-mantle conveyor belt (Fig. 4). The suggested structure (Fig. 5) is very similar to a first approximation to the 'infinite onion' model first proposed by Cann⁶. In fact, the details of the reconstructed profiles in the Bay of Islands complex are even more onion-like than those of Cann's theoretical model, however, the genetic distinctions made by Cann between cumulate, layered gabbro and isotropic, plated gabbro do not seem to apply here.

The magma chamber profiles and major lithologic units of the plutonic section observed in the Bay of Islands complex seem to be continuous and systematic and to lack large-scale truncations. This strongly suggests that a steady-state magma chamber was present. The longest axis of the chamber was parallel to the ridge axis; its ultimate length being limited by its truncation at fracture zone intersections. The minimum height of the magma chamber can be estimated from the thickness of the plutonic group to be ~6 km. While allowing for possible rotation of unit contacts in the structurally deepest regions due to the motion of the underlying residual upper mantle, reasonable estimates of maximum magma chamber height based on the North Arm Mountain structure would range from 6 to 8 km if measured at the precise ridge axis. Assuming bilateral symmetry, the width of the chamber could have been as much as 14–16 km based on the length of the clinopyroxenite megalens and the trajectory of fine-scale igneous layering in the overlying transition zone and gabbroic unit. Such a large magma chamber is not inconsistent with theoretical thermal calculations^{5,42} and seismic results^{2,3,14} for mid-ocean ridges with fast spreading rates.

The magma chamber profiles described above appear to be quite variable as layering trends are often very irregular locally. Even in map areas adjacent to those described here similar large-scale arcuate structures are not well defined. These areas are characterized by highly variable discordant layer orientations and may reflect considerable unevenness in the morphology of the chamber walls in time and space. Some of these irregularities might evolve due to magma current erosion and/or the growth of reef-like protrusions formed by localized rapid growth at points along the walls²⁵. Localized fracturing and cooling of the crust by hydrothermal circulation might also cause cold spots along chamber walls where crystallization might occur at an accelerated pace.

In the Lewis Hills massif, an arcuate profile oriented approximately perpendicular to those in the northern massifs exists. This structure is interpreted as the result of vertical pinching-out

Fig. 5 Schematic models for the formation of oceanic lithosphere depicted by cross sections oriented perpendicular to a fast (10 cm yr^{-1}) half spreading rate (a) and a slow (2 cm yr^{-1}) spreading rate (b). Shading as in Fig. 2. *a* is based on the geology and approximate unit thicknesses observed for the North Arm Mountain section. An intrusive/extrusive carapace of pillow lava, diabase and some underplated gabbro floating over magma chambers of different widths and different shapes are depicted. Chambers are situated over the diverging limbs of depleted upper mantle. Bold dashed lines indicate $1.25 \times 10^5 \text{ yr}$ isochrons. Bold solid lines depict the shapes of the steady-state magma chamber at various times in the past as determined from the arcuate patterns of fine-scale igneous layering (thin dashed lines). Note that the fine-scale igneous layering (or time lines) strike obliquely across the major lithologic subdivisions of the plutonic section reflecting chemical zonation of the magma chamber. Flow lines for the upper mantle and overlying crust are depicted by dashed lines in the residual mantle unit. Sea floor topography depends on the equilibrium size of the magma chamber⁴⁵ which is ultimately determined by the spreading rate^{5,42}. The final fine-scale igneous layering geometry is determined by the size and shape of the former magma chamber.



of the magma chamber where it was truncated at a ridge-ridge transform fault. The structure seems to indicate that the lower surface of the magma chamber shallowed considerably at this intersection while the seafloor presumably deepened. Assuming no major change in the level of the top of the harzburgite unit, a relatively thin overlying gabbroic unit is inferred (Fig. 4b). Overall the structure suggests decreasing height of the magma chamber as the fracture zone intersection is approached. Lateral dimensional changes cannot be clearly resolved in the preserved layering geometry. Very irregular layering, abundant gabbro xenoliths, and periodotite plutons obscure the details of the layering in the central part of the Lewis Hills massif along the preserved magma chamber truncation. The increase in relative thickness of ultramafic cumulates in addition to the shallowing of the chamber floor could result in progressive shallowing of the Moho near fracture zones as documented by recent marine seismic refraction studies⁴³.

Model of plate accretion

A mid-ocean ridge environment represents a site of mantle upwelling as well as mantle divergence⁴⁴ in which the mantle flow direction makes a right angle turn from vertical beneath the ridge to horizontal on its flanks. In such a case the 'frozen in' mantle flow lines will be only moderately inclined to be horizontal on the ridge flanks. When viewed on a large scale this upwelling can be modelled in terms of divergent flow of a viscous fluid⁴⁵. As in any such viscous flow, the mantle flow rising and diverging beneath an oceanic ridge follows smooth curved paths. A sharp right angle turn of the flow lines is not possible because it would require infinite acceleration at the inflection point.

Nelson⁴⁵ has suggested that the surface of the upwelling mantle must, in effect, define an axial valley of some finite width and has modelled the variable topography along mid-ocean ridges in terms of mantle divergence and the equilibrium size of the sub-axial magma chamber.

The structural data from the tectonites of the upper harzburgite section and the lowermost plutonic section of the Bay of Islands complex are consistent with the mantle upwelling model. The tectonite lineation direction, interpreted to lie close to the plastic flow direction, is subparallel to palaeohorizontal surfaces and subperpendicular to the mean dyke orientation. Both the foliation and lineation have been 'frozen' into a subhorizontal position which lies close to the predicted flow direction in the mantle⁴⁶⁻⁴⁸.

The contact between the rocks of the plutonic group and the residual harzburgite of the ophiolite which seems to be sub-horizontal represent an unconformity between rocks of magmatic origin and depleted upper mantle origin. The existence of this sharp contact requires the surface of residual mantle to intersect the base of the crustal level magma chamber at some time⁸. The most likely place for the creation of this contact is near the axis of divergence where the 'surface' of the upwelling residual mantle is, in essence, continually being created.

Because the lowermost plutonic section is affected by the same high temperature ductile deformation that affects the residual harzburgite, internal deformation must occur here while accretion of the plutonic section onto the surface of the upwelling mantle proceeds. The flow lines in the overlying plutonic rocks will grossly parallel the flow lines of the surface of the upwelling mantle as they are progressively accreted, while the carapace of the magma chamber which probably has a bulk

density equal to or less than the magma below²⁷ simply 'floats' on top of it. The carapace of the chamber, and therefore the sea floor, will not necessarily mirror the surface of the upwelling mantle until the entire thickness of the crust has solidified at the outermost extremity of the magma chamber. The equilibrium size of the magma chamber, largely dictated by spreading rate^{5,42}, will determine the ridge topography⁴⁵ (Fig. 5).

From the point of formation of the surface of the mantle (the axis of divergence) the plutonic section will gradually be accreted and thicken largely by heterogeneous nucleation and *in situ* crystallization at the bounding surface of the magma chamber, until at the outer margin of the steady state chamber the crust is completely solidified. The layer pattern preserved in plutonic section will outline the former surfaces of the chamber and therefore represent isochrons. Identification of a pattern will depend on regularity in the shape of the chamber, and if delineated, will conform to the size and shape of the magma chamber. The shape and size defined by the layering pattern is unlikely to be the same in every ophiolite and in the simplest case the equilibrium size of the chamber ultimately depends on spreading rate^{5,42}. Therefore, an estimate of relative spreading rate within ophiolites may be made using the chamber dimensions defined by these profiles. Where spreading rates are very slow or where non-steady-state episodic spreading occurs magma chambers may become so narrow that they periodically solidify completely. Large-scale truncations of the layered

structure and lithologic units would be expected to result in such cases. We see no such evidence at present in the Bay of Islands complex. Given this and the observed dimensions inferred from the layering profiles, a relatively fast spreading history is inferred. High temperature ductile deformation of the lower plutonic section in some cases may obscure the original angle of intersection.

Regular well-preserved and ordered structures such as those described here may be rare in ophiolite complexes. However, identical patterns have been described for the Semail ophiolite⁴⁹. On the other hand, if such relations have been overlooked or distorted by forcing all cumulate layering into a reconstructed subhorizontal orientation, as is commonly required, serious errors in layer thickness estimates could result. This might partially account for small apparent thicknesses of ophiolites relative to the seismic thickness of the oceanic crust¹².

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Structure of the actin-myosin interface

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The topography of the rigor complex between F-actin and myosin heads (S1) has been investigated by carbodiimide zero-length cross-linking. The results demonstrate for the first time that the 95,000-molecular weight (95K) heavy chain of the myosin head enters into van der Waals contact with two neighbouring actin monomers; one is bound to the 50K domain and the other to the 20K domain of the myosin chain. The covalent F-actin-S1 complex can be isolated; it shows a vastly elevated Mg^{2+} -ATPase. Each pair of actin subunits in the thin filament seems to act as a functional unit for specific binding of a myosin head and stimulation of its Mg^{2+} -ATPase activity.

MUSCULAR contraction and cell motility are thought to operate by a cyclic displacement of myosin heads (S1 'cross-bridges') along the actin filaments. The mechanical force is

generated at the myosin-actin interface and is coupled to Mg^{2+} -ATP hydrolysis catalysed by the actin-myosin complex. The mechanism of the interaction is poorly understood.

The aims of the work reported here were to identify the participating elements and elucidate the main structural features

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of the actin-myosin head interaction region and to establish the relationship of this van der Waals complex to actin activation of the myosin ATPase activity and cross-bridge function. Using a chemical cross-linking reaction as a molecular probe, we have investigated the mode of attachment of S1 heavy chain to F-actin in a variety of experimental conditions, and have identified the covalently associated actin-S1 entities formed. We have also determined the enzymatic properties of the myosin heads consequent on their irreversible attachment to F-actin in defined conditions.

Experimental strategy

Our experiments were based on three critical parameters. (1) Cross-linking reactions were performed on rigor complexes of F-actin using the following: native chymotryptic S1 (A1 + A2), S1 (A1), S1 (A2)¹ and the three tryptically fragmented S1 derivatives²⁻⁴, the heavy chain of which is a complex of two or three fragments: (27K-50K-20K) (molecular weights 27,000-50,000-20,000)-S1, (27K-70K)-S1 and (75K-22K)-S1. These proteolytic S1 derivatives result from restricted tryptic hydrolysis of S1 in the absence and presence respectively of F-actin, which specifically modulates the breakdown of a 2K segment linking 50K and 20K fragments and confers protection against loss of actin-S1 Mg^{2+} -ATPase³. All these derivatives interact reversibly with actin. The fragmented nature of their heavy chain allows direct identification of the parts of the chain which are engaged in cross-linking with actin. In preliminary experiments, cross-linking reactions were also carried out using heavy meromyosin (HMM)¹.

(2) Before cross-linking, rigor actin-S1 complexes were prepared, in which either the actin or the S1 (or its derivatives) were labelled with the fluorescent dye *N*-(iodoacetyl)-*N'*-(1-sulpho-5-naphthyl)ethylenediamine (1,5-IAEDANS), the actin at cysteine 373 and the S1 at SH₁ thiol group³. This

procedure allowed direct identification of actin and 20K peptide-containing material (SH₁ is located in the 20K unit⁵) in the observed cross-linked products. The molar ratios of actin/S1 present in cross-linked species were estimated quantitatively. This was done by measuring the ³H/¹⁴C ratio in the species resulting from cross-linking reactions performed on complexes of actin and S1, labelled at the sites mentioned above with ³H-iodoacetic acid and ¹⁴C-iodoacetamide, respectively.

(3) Cross-linking reactions were catalysed with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), a water-soluble carbodiimide that forms zero-length covalent bonds between amino and carboxyl groups in the contact area between two proteins. Most experiments were done using our highly versatile two-step procedure. First, actin was treated with EDC (activation phase), then an aliquot of the activated protein was mixed with a solution of S1, to give at least a fivefold dilution of the actin (condensation phase). This second step ensures a simultaneous quenching of the initial EDC reaction and covalent association of the two proteins. This procedure avoids most of the disadvantages of the usual one-step method; it suppresses intramolecular cross-linking when fragmented S1 or double-headed HMM are used, it allows the two phases of the reaction to be carried out separately in specific optimal conditions, and it also permits the controlled EDC-activation of either actin or S1 at will, and thus affords information on the compositions of both proteins at the interface.

Specific binding of 95K heavy chain to two vicinal actin subunits

Analysis of the products of reaction of EDC-activated actin with S1 (A1 + A2) by gradient SDS-polyacrylamide slab gel electrophoresis gave the protein band pattern shown in Fig. 1A. It is characterized by the presence of two new components, both present as doublets. Estimations on calibrated 5-18% acrylamide gels indicated that the major species had *M_s* of ~180,000 (185,000 and 175,000 for the components of the doublet), while that of the other, minor species was 265,000 (260,000 and 270,000). The distribution of the fluorescence in the protein bands formed from EDC-treated rigor complexes containing either fluorescent actin or fluorescent S1 demonstrated unequivocally that the two protein doublets were covalently cross-linked adducts of actin and S1, as label from either source was incorporated (Fig. 1B, C). They were sometimes accompanied by traces of two components which were fluorescent only when fluorescent actin was used and which were found in the EDC-actin control (Fig. 1D). Their electrophoretic mobility is consistent with their assignment as intramolecularly cross-linked actin dimers and trimers. The exact origin of the actin-S1 protein doublets is unknown but it does not arise from the heterogeneity associated with the presence of heads bearing different alkali light chains, as similar results were obtained with purified S1 (A1) and S1 (A2).

While the minor 265K product is sometimes absent, the major 180K derivative is always abundant in a wide range of experimental conditions (pH 6-7.5 at 0-20 °C and ionic strength 0.01-0.1 M in non-nucleophilic buffer). In optimal conditions ~30% of S1 becomes covalently bound to F-actin as determined by sedimentation (see below). However, no cross-linking occurs when activated actin is reacted with S1 in the presence of Mg^{2+} -pyrophosphate or Mg^{2+} -ATP (5 mM), which are known to dissociate the actin-myosin complex, and more importantly, no covalent association can be obtained between native actin and EDC-activated S1 (although the reagent does react appreciably with S1 carboxyl groups in the standard conditions of the activation reaction). The absence of covalent links between activated S1 and actin is not due to any effect of EDC on the reversible association between S1 and actin, as the 180K product is readily formed when the cross-linking reaction is done using the conventional one-step procedure. Therefore the 180K complex seems to be the result of a very specific cross-linking process which requires the formation of a bimolecular

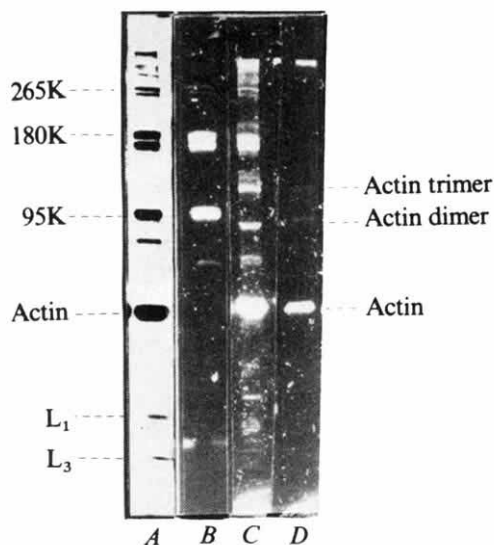


Fig. 1 SDS-gel electrophoretic analysis of actin-S1-containing cross-linked products. F-actin purified according to Spudich and Watt¹⁸ was washed and equilibrated by sedimentation in 100 mM 2-N-morpholinoethanesulphonic acid (MES), pH 6.0, then immediately incubated (2.5 mg ml⁻¹) with 15 mM EDC (added as a solid) in the MES buffer for 2 min at 20 °C. A sample aliquot was mixed with 5-10 volumes of S1 solution in the same buffer (final molar ratio of actin/S1 = 2). After 10 min at 20 °C, samples (≈35 μg S1) were submitted to SDS-polyacrylamide slab gel electrophoresis (5-18% gradient acrylamide) using a 50 mM Tris/100 mM boric acid buffer system³. Gels were viewed using long-wave UV light and stained with Coomassie blue. Before use, F-actin or S1 was made fluorescent with 1,5-IAEDANS according to Takashi¹⁹ and Duke *et al.*²⁰, respectively. **A**, protein bands stained with Coomassie blue after reaction of S1 with EDC-activated actin; this pattern is unchanged when labelled S1 or labelled actin is used. **B**, fluorescent bands produced by reaction of IAEDANS-S1 with activated F-actin. **C**, fluorescent species produced by reaction of S1 with activated IAEDANS-actin. **D**, pattern of fluorescence in the EDC-activated IAEDANS-actin control. No change in S1 pattern was observed when the cross-linking reaction was processed in the absence of actin.

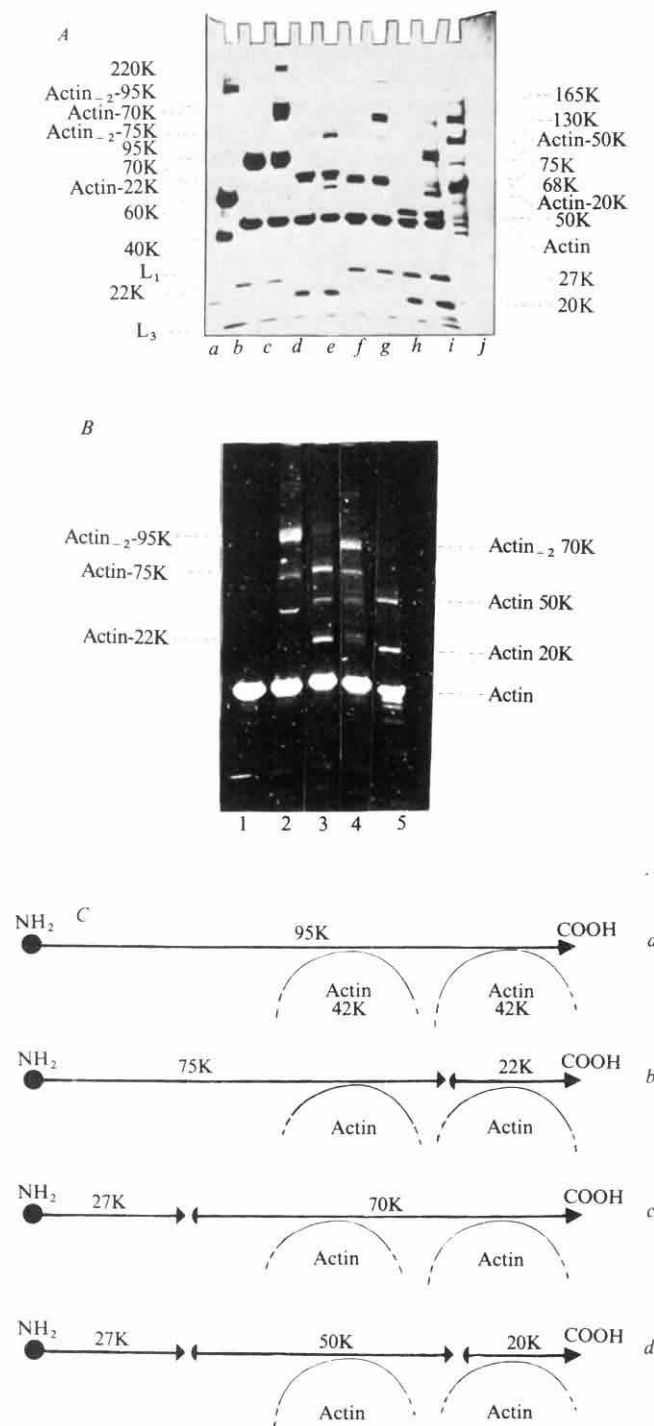


Fig. 2 Mapping of the heavy chain segments bound to the actin subunits by cross-linking the trypsin-modified S1 derivatives to fluorescent F-actin. The tryptically fragmented S1 species were prepared as described elsewhere⁴ and condensed to EDC-activated IAEDANS-actin as described in Fig. 1 legend; the same procedure was followed for native S1. Samples were analysed by electrophoresis on 5–18% acrylamide slab gels. **A**, protein banding pattern after staining with Coomassie blue. Lanes *a* and *j*, protein markers: ferritin (M_r 220,000), γ subunit of *Escherichia coli* RNA polymerase (165,000), β -galactosidase (130,000), bovine serum albumin (68,000), catalase (60,000), aldolase (40,000). Lanes *b* and *c*, native S1 + labelled actin before and after cross-linking. Lanes *d* and *e*, labelled actin + (75K–22K)–S1 before and after cross-linking. Lanes *f* and *g*, labelled actin + (27K–70K)–S1 before and after cross-linking; *h* and *i*, labelled actin + (27K–50K–20K)–S1 before and after cross-linking. **B**, location on gel of fluorescent actin-containing cross-linked products. Lane 1, fluorescent actin control; 2, fluorescent actin cross-linked to native S1; 3, fluorescent actin cross-linked to (75K–22K)–S1; 4, fluorescent actin cross-linked to (27K–70K)–S1; 5, fluorescent actin cross-linked to (27K–50K–20K)–S1. **C**, schematic diagram illustrating the relationship of the two actin subunits to the segments of myosin head heavy chain. *a*, Native S1; *b*–*d*, tryptically fragmented S1 derivatives.

Table 1 Estimation of the actin/S1 molar ratio present in the cross-linked 180K product

Experiments	³ H-actin/ ¹⁴ C-S1 (molar ratio)
1	2.5
2	1.6
3	2.3
4	1.4
5	2.6
6	2.4
7	1.5

³H-carboxymethyl actin (prepared according to Takashi *et al.*²³) (~2.0 ³H per mol protein, 18 mCi mmol⁻¹) was activated with EDC and reacted with ¹⁴C-carboxymethyl S1 (obtained by the same procedure: ~1.5 ¹⁴C per mol protein, 45 mCi mmol⁻¹) as described in Fig. 1 legend. Samples (~100 μ g S1) were subjected to SDS-polyacrylamide gel electrophoresis (4–18% acrylamide gradient). After staining with Coomassie blue, the 180K protein doublet was isolated as a gel slice; two slices were combined and treated with 1 ml of Soluene 100 at 40 °C for 4 h. Radioactivity was measured in 10 ml of scintillator using a Packard 3003 counter. Contribution of ³H to ¹⁴C counts was subtracted using ³H–¹⁴C calibration mixtures. The data shown are from different cross-linking experiments.

complex in which EDC-activable carboxyl groups on the actin are in contact with nucleophilic, probably amino groups on the myosin heads. This conclusion agrees with the suggestion made by Bárány and Bárány⁶ that the actin–myosin complex is a polyelectrolytic system in which the actin behaves as a polyanion and the myosin as a polycation. In addition, the ionic strength dependence of the rate constant of association of actin with S1 (ref. 7) indicates an ion-paired charge interaction at the recognition site.

The apparent mass of the 180K species containing both S1 and actin suggested that it must be generated by the association of two actin monomers with a single 95K heavy chain. The first direct evidence for this composition was obtained by determining the ³H/¹⁴C ratios of the 180K protein doublet formed by cross-linking ³H-actin to ¹⁴C-S1. The results showed an average ratio of ~2 which indicates a stoichiometry of two actin subunits per head (Table 1). Peptide mapping of the cross-linked species formed by complexes of fluorescent actin with the three tryptically fragmented S1 derivatives (Fig. 2A, B) not only confirmed this result but also led to the important conclusion that the two actin subunits are not associated by intermolecular EDC-induced cross-links but rather are separately and specifically bound to two different segments of the 95K heavy chain, that is, 50K and 20K fragments. Thus (75K–22K)–S1 gives rise to two new fluorescent cross-linked products, actin–75K peptide (M_r = 120,000; 120K) and actin–22K peptide (M_r = 64,000) (Fig. 2A, lanes *d* and *e*; B, lane 3). When (27K–70K)–S1 is used, the two actin subunits are both bound to the intact 70K segment to form the new cross-linked species (actin₂–70K peptide (M_r = 155–160,000), Fig. 2A, lanes *f* and *g*; B, lane 4). The two actin-binding domains present in (27K–50K–20K)–S1 can be characterized by their ability to cross-link to the pair of actin monomers, yielding actin–50K peptide (M_r = 94,000) and actin–20K peptide (M_r = 62,000) (Fig. 2A, lanes *h* and *i*; B, lane 5). Interestingly, only the actin₂–70K peptide complex, containing two actin monomers, yields a doublet on the gel; all the other species, including actin–75K peptide, contain a single actin subunit and migrate as single bands. This suggests that the observed electrophoretic heterogeneity may be due to the presence of the pair of actin molecules. We have confirmed the distribution of the actin pair between the various heavy chain segments (see Fig. 2C) by repeating the above observations with fluorescently fragmented S1. Moreover, when the reaction mixture of EDC-activated fluorescent actin–S1 was treated with trypsin², a large amount of actin₂–70K fragment was liberated, together with smaller proportions of actin–20K peptide and actin–50K peptide, which indicates that the actin subunits are dispersed in a similar manner on intact S1 and its fragments. We have failed to observe a cross-linked species corresponding to

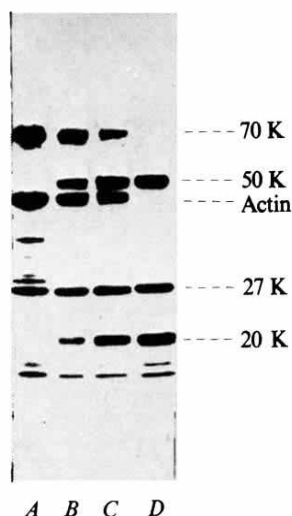


Fig. 3 Relationship between S1 binding to actin and the tryptic sensitivity of the 95K heavy chain (at low ionic strength, excluding any effect of salt on the S1-actin binding affinity). Tryptic conversion of actin-bound S1 into actin-activable (27K-70K)-S1 and actin-nonactivable (27K-50K-20K)-S1 derivatives. Samples A, B and C were analysed by electrophoresis as described in Fig. 1 legend; S1 fragmented in the absence of actin was used as control (lane D). Molar ratios of actin/S1 present in the digestion mixture were A = 2, B = 1 and C = 0.5.

the direct association between a single actin monomer and the intact or fragmented 95K heavy chain.

A cross-linking study was carried out at different actin/S1 molar ratios (0.5–10) to investigate conditions which favour the formation of the 265K derivative; this showed that the amount of the 180K component alone increases with increasing concentration of actin, reaching a maximal value at an actin/S1 molar ratio near 2. In contrast, yield of the cross-linked 265K component remains rather low even at an actin/S1 ratio of 1, although further studies (see below) showed that all S1 molecules are bound to actin at this ratio. Thus we assume that rigor attachment of an actin pair to the first S1 molecule imposes a different geometry on the binding of the second S1 molecule. This difference emerges from the capacity of the activated actin carboxylates to cross-link the first S1 compared with the much less efficient attachment of the second S1 at the topologically equivalent second site. This is indicative of the steric non-equivalence of the two heads in the complex.

Strong support for this scheme comes from a parallel study undertaken to define the relationship between the tryptic sensitivity of the 50K-20K junction of S1 heavy chain and the relative amount of actin needed to protect it. The actin-S1 ATPase activity, which depends on the structural integrity of the 50K-20K join³, is insensitive to trypsin at actin/S1 molar ratios ≥ 2 . Below a ratio of 2 it decreases, and at a ratio of 1, when all S1 molecules are bound to actin as assessed by turbidimetry and sedimentation⁸, ~50% of this enzymatic activity is lost. The gel electrophoretic pattern of the tryptic digest of an equimolar mixture of actin and S1 (Fig. 3) clearly indicates the formation of two types of S1 derivative in equimolar amounts: (27K-70K)-S1 (ATPase activity activable by actin) and (27K-50K-20K)-S1 (ATPase activity not actin activable). At the critical molar ratio of actin/S1 = 2, only the former active derivative is present. Thus trypsin is able to discriminate between the two kinds of S1 bound to the same actin dimer unit, these being the 'trypsin-sensitive' and 'trypsin-insensitive' species (Fig. 5). We obtained similar results with both S1 and myosin in other ionic conditions³. In this regard, the tryptic susceptibility of the 50K-20K junction of S1 heavy chain seems to be an excellent probe of the mode of attachment of the head to actin. It indicates also that the

protective action of F-actin towards the 50K-20K join^{2,3} is not due to the simple binding of S1 to actin but rather requires a specific actin-head recognition interface generated by the association of an actin dimer with a single S1 molecule in the absence of nucleotide. This mode of actin-head rigor interaction probably also occurs *in vivo*, as suggested by the findings of Lovell and Harrington⁹ on the tryptic susceptibility of rabbit skeletal myofibrils in physiological rigor conditions.

Covalent F-actin-S1 complex

Because the actin- and nucleotide-binding sites in myosin are coupled¹⁰, we have examined the effect of covalent association of S1 with actin on its Mg^{2+} -ATPase activity. We found that the Mg^{2+} -ATPase activity was greatly increased, and Fig. 4A, B shows that the extent of the enhancement depends on the time courses of the activation of the actin by EDC and of the attachment of S1 to the activated actin. The extent of Mg^{2+} -ATPase activation is apparently related to the amount of 180K product formed; it increases with increasing actin concentration and reaches a plateau at an actin/S1 molar ratio near 2. Conditions (see above) which suppress the 180K species also eliminate the ATPase activation. Control experiments have shown that P_i liberation is not induced by the small amount of free actin accompanying S1 in the ATPase assay; in addition, this ATPase, in contrast to that of the reversible actin-S1 complex, is not affected by increased ionic strength. Finally, a parallel decrease in K^+ -ATPase of the EDC-activated actin-S1 mixture accompanies Mg^{2+} -ATPase stimulation (Fig. 4B). We conclude that only the fraction of S1 that is covalently bound to actin is responsible for this new enzymatic behaviour and is able to catalyse massive hydrolysis of Mg^{2+} -ATP. This is demonstrated by ultracentrifugal fractionation of the EDC reaction mixture in the presence of Mg^{2+} -pyrophosphate into soluble and insoluble S1-containing fractions. The supernatant contains only residual S1 (Fig. 4C, lane 2) with unmodified enzymatic activity, but the pellet consists of irreversibly actin-bound S1 (Fig. 4C, lane 3) and shows elevated Mg^{2+} -ATPase, the K^+ -ATPase being close to zero. The former activity remains unchanged when assayed in the presence of an added excess of free actin.

On the basis of the amount of S1 covalently linked to actin, estimated spectrophotometrically from the concentration of the enzyme in the supernatant⁸, we have calculated the specific Mg^{2+} -ATPase activity to be $22 \pm 2 \mu\text{mol } P_i$ per min per mg actin-bound S1, which corresponds to a turnover rate of 40 s^{-1} . When compared with the initial value of $0.02 \mu\text{mol } P_i$ per min per mg actin-bound S1 (turnover rate = 0.036 s^{-1} in agreement with reported values¹), this represents at least a 1,000-fold increase in the ATPase; the turnover rate of the covalent actin-S1 complex is strikingly similar to the V_{max} of the usual actin-activated ATPase of S1 (A1 + A2) extrapolated to infinite actin concentration¹. We refer to this unprecedented acceleration of Mg^{2+} -ATP hydrolysis catalysed directly *in vitro* by actin-bound S1 as 'superactivation', to differentiate it from any other activation process observed for this myosin activity *in vitro*.

Conclusions and implications

On the basis of these results we conclude that each actin monomer has two heavy chain-binding regions, specific for the 50K and 20K elements, respectively. The myosin head-binding unit of the thin filament in rigor conditions would thus be a contiguous actin pair. Consequently each functional pair of neighbouring actin monomers must have two pairs of head sites allowing the stoichiometric binding of a pair of heads (Fig. 5). This is supported by the occurrence of the 265K species which comprises actin and S1 and whose apparent mass agrees with its identity as the product of a pair of 95K heavy chains cross-linked with a pair of actin molecules.

Our results agree with the model recently proposed by Namba *et al.*¹¹ for the thin filament structure of crab striated muscle

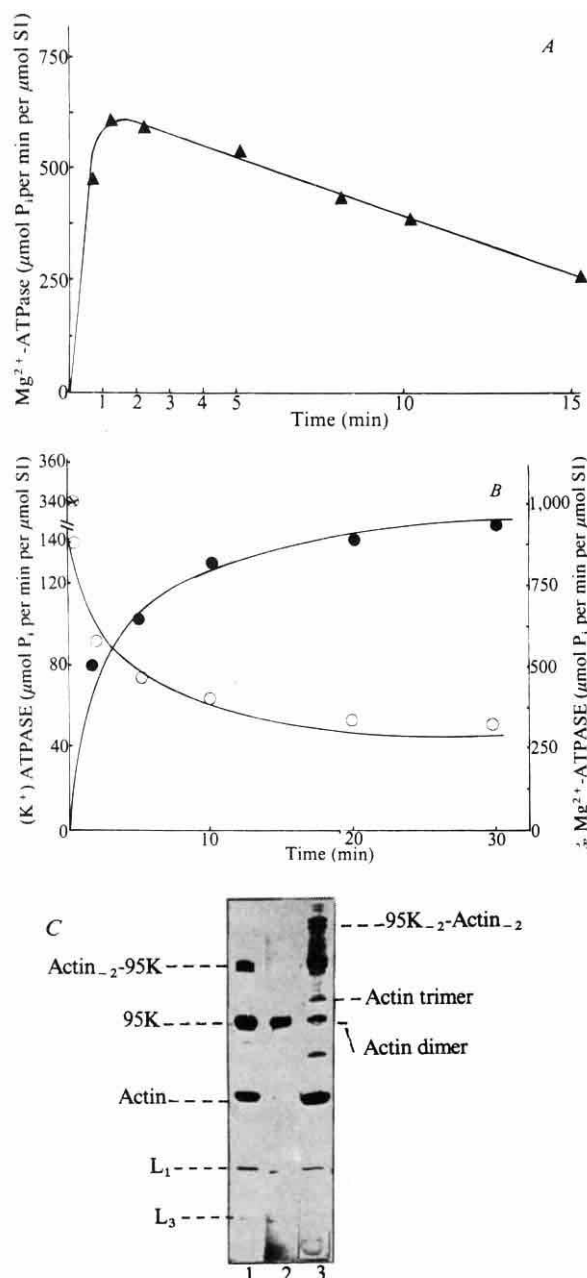


Fig. 4 Superactivation of the Mg^{2+} -ATPase of S1 covalently bound to EDC-activated F-actin. **A**, dependence of the Mg^{2+} -ATPase stimulation on the time course of actin activation by EDC. F-actin (2.5 mg ml^{-1}) in 100 mM MES buffer, pH 6.0, was reacted at 20°C with 15 mM EDC (added as a solid). At the times indicated, a protein aliquot was withdrawn and mixed with 10 volumes of S1 solution in the same buffer (final molar ratio of actin to S1 = 2); after 10 min of condensation reaction at 20°C , samples ($20 \mu\text{g}$ S1) were assayed for Mg^{2+} -ATPase²¹. **B**, dependence of the Mg^{2+} -ATPase stimulation and K^{+} -ATPase inhibition on the time course of S1 condensation to EDC-activated actin. F-actin was first activated with EDC for 2 min, then mixed with S1 in conditions described above for **A**. At different times in the condensation reaction, S1 samples, $20 \mu\text{g}$ and $25 \mu\text{g}$, were taken in parallel and assayed for Mg^{2+} -ATPase and K^{+} -ATPase, respectively²¹. ●, Acceleration of Mg^{2+} -ATPase activity, and ○, loss of K^{+} -ATPase activity of S1 covalently bound to actin. As reversible binding of S1 to actin inhibits the K^{+} -ATPase at zero time of the cross-linking reaction²², the K^{+} -ATPase was assayed in the presence of actin (actin/S1 ratio = 2); the value found was 60% lower than that given by free S1 (⊗). **C**, selective separation of the covalent actin-S1 complex. After a 10-min period of condensation between activated actin and S1, the reaction medium was adjusted to 10 mM sodium pyrophosphate, 100 mM KCl, 5 mM MgCl_2 and 50 mM HEPES pH 7.5; after centrifugation at $140,000g$ for 45 min at 4°C , the pellet was gently resuspended in the initial volume of a solution containing 10 mM KCl, 1.5 mM MgCl_2 and 50 mM Tris-HCl pH 8.0. It was subjected, together with the supernatant, to electrophoresis on gradient acrylamide gel as described in Fig. 1 legend. Lane 1, total reaction medium; lane 2, supernatant containing unreacted dissociable S1; lane 3, pellet containing nondissociable S1, covalently bound to the actin filament as major actin₂-S1₁ and minor actin₂-S1₂ species.

analysed by X-ray diffraction, which showed myosin heads attached to pairs of adjacent actin monomers. Actin-20K peptide and actin-50K peptide are now being isolated for elucidation of the points of contact between actin and the two heavy chain segments. Experiments in progress have also indicated EDC-induced cross-linking of actin dimers to the heavy chains of HMM, with concomitant superactivation of the Mg^{2+} -ATPase. These data, together with those for the parent myosin, will be reported in detail elsewhere; the use of myosin is of particular interest because it contains molar amounts of LC2 light chain, the presence of which is reported to change the shape of the attached heads¹².

The covalent actin-S1 complex hydrolyses Mg^{2+} -ATP at a very high rate because of the irreversible occupation of the actin site on S1. This provides the first direct experimental evidence for the possible non-dissociating pathway of ATP hydrolysis by actin-S1^{13,14}. Cross-linking of actin to S1 does not impair the catalytic properties of the enzyme but, on the contrary, preserves the active conformation of the ATPase site resulting from the interaction of actin with S1. The covalent actin-S1 material offers a valuable tool for investigating the kinetic parameters of ATP hydrolysis by actin-bound S1 and for determining the nature of the conformational changes transmitted to the ATPase site when the actin site is bound.

The ability of S1 covalently complexed to actin to hydrolyse ATP at an effective rate as high as that which normally occurs in the presence of infinite actin concentration should be considered in relation with the report of Bárány and Burt¹⁵ which indicates that *in vivo* contraction is associated with 1,500–5,000-fold activation of myosin ATPase, which has never been seen in any *in vitro* system. Our covalent actin-S1 derivative may represent the first assembly *in vitro* of interacting actin-head complex resembling that found in the native cell.

The rigor association of S1 heavy chain with an actin dimer seems to be closely related to the molecular mechanism of stimulation of myosin Mg^{2+} -ATPase by F-actin. Estes and Gershman¹⁶ have shown that neither G-actin nor F-actin monomers can activate the ATPase; they have concluded that the specific activating ability of F-actin is possibly formed or is stabilized by the association of two adjacent subunits. In addition, Fasold *et al.*¹⁷ have reported that isolated, non-polymerizable actin dimers generated by chemical cross-linking of F-actin bind stoichiometrically to S1 and activate the ATPase

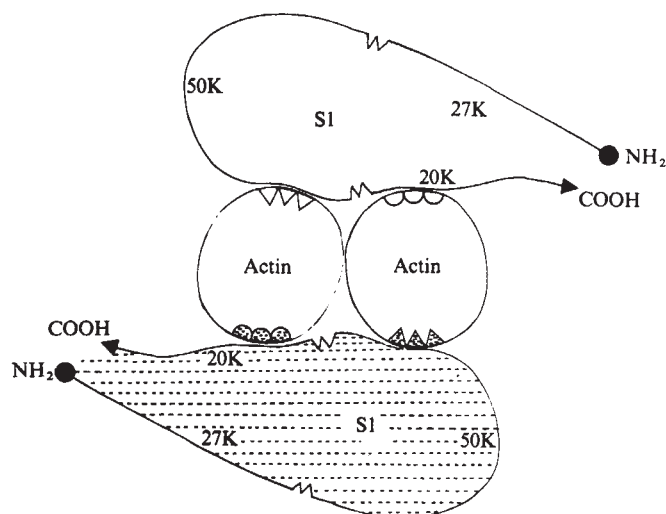


Fig. 5 Schematic diagram for stoichiometric binding of S1 to two actin monomers which are on two strands of the actin helix: hatched S1 is only poorly cross-linked, trypsin-sensitive S1; open S1 is strongly cross-linkable, trypsin-insensitive S1 (tryptic sensitivity is implicitly related to the splitting of the 50K-20K join).

25% relative to F-actin. Our results strongly support all these observations and further suggest that the proper positioning of the actin pair on the 50K and 20K segments of the heavy chain is a prerequisite for, at least, the initiation of the ATPase activa-

tion process.

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Gene conversion between duplicated genetic elements in yeast

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The mitotic recombination behaviour of a duplication of the his4 region on chromosome III in the yeast Saccharomyces cerevisiae was studied. The major recombination event between the duplicated segments is gene conversion unassociated with reciprocal recombination. The rad52-1 mutation preferentially decreases mitotic gene conversion. These results suggest that mitotic gene conversion may occur by a different pathway from that occurring in meiosis. This mitotic gene conversion may be important in yeast mating type interconversion and the maintenance of sequence homogeneity in families of repeated eukaryotic genes.

GENETIC recombination is the result of an exchange between homologous regions of DNA and can occur between two segments located on homologous chromosomes, or between repeated segments of DNA on the same chromosome. Two types of homologous recombination have been observed in yeast and other organisms; reciprocal and non-reciprocal exchange, or gene conversion (reviewed in refs 1–3). In yeast, meiotic gene conversion is associated with reciprocal recombination⁴; as many as half the conversion events are accompanied by reciprocal recombination of flanking markers, and this conversion-associated recombination is frequent enough to account for all the genetic exchange observed in yeast meiosis. Current models for meiotic recombination postulate an exchange of DNA strands between homologous DNA duplexes^{1–3,5,6}. If there are base sequence differences between the two duplexes, the resulting structure contains a local region of heteroduplex. Gene conversion is believed to be due to the correction of the heteroduplex region. These models postulate that resolution of the heteroduplex results in recombination of flanking markers in 50% of cases.

Increasing evidence suggests that mitotic recombination may occur by different pathways from those of meiotic recombination. In mitosis, gene conversion is not as closely associated with reciprocal exchange^{7,8}. Roman⁷ found that UV-light irradiation enhanced mitotic reciprocal recombination more than gene conversion, and that nitrosoguanidine enhanced gene conversion preferentially. In addition, Boram and Roman⁹ have isolated an allele of *rad18* that enhances spontaneous mitotic gene conversion, but not reciprocal recombination. A further difference between mitotic and meiotic recombination in yeast is seen in the effects of other mutations. The *rem1-1* mutation enhances both types of exchange in mitosis, but has no effect on meiotic recombination^{10,11}. In contrast, the *rad52-1* mutation

abolishes meiotic recombination, but only decreases mitotic exchange^{12–14}. Thus, in mitosis, gene conversion and reciprocal recombination can be separated from each other. These considerations suggest that there may be mechanistic differences between meiotic and mitotic recombination.

We have used hybrid plasmids containing yeast DNA linked to an *Escherichia coli* plasmid to study mitotic recombination. During transformation these hybrid plasmids integrate by a homologous recombination event and create a direct duplication of the yeast DNA separated by plasmid sequences¹⁵. We have constructed a direct duplication of a 24-kilobase (kb) *Bam*HI fragment on chromosome III which contains the *his4* gene. By genetic techniques, different mutations were introduced into each of the two copies of the *his4* gene, creating a duplication with the structure *his4A*[–] pBR313 *his4C*[–]. Mitotic recombination between the two *his4* mutant genes can yield a *HIS4*⁺ recombinant. Whether the event which generated the recombinant was gene conversion or reciprocal recombination can be determined by analysis of the recombinants. A reciprocal

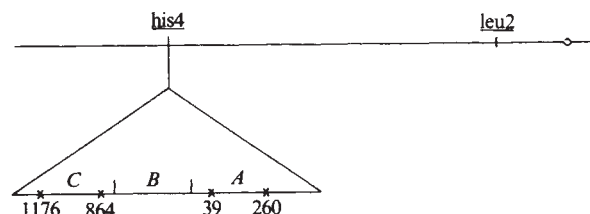
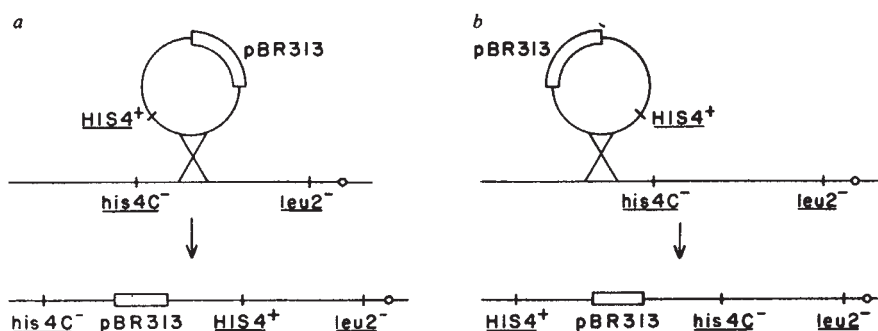


Fig. 1 Map of chromosome III. The *his4* region is shown subdivided into A, B and C. The relevant *his4A* and *his4C* mutations are shown.

Fig. 2 Formation of duplication strains by transformation. The plasmid YIP300, which consists of a *Bam*HI fragment carrying the *HIS4*⁺ gene inserted into pBR313, is shown to integrate into the yeast chromosome III by a homologous recombination event. The recombination event can occur centromere-proximal or centromere-distal with respect to the *his4C*⁻ mutation on chromosome III. *a*, Orientation of the two *his4* genes if the exchange event occurs proximal to the centromere; *b*, orientation of the two *his4* genes if the exchange event occurs distal to the centromere. The line represents yeast DNA and the box represents pBR313 sequences.



recombination event gives rise to a *HIS4*⁺ segregant which has lost the duplication and the intervening plasmid sequences (see Fig. 4). A gene conversion event between the two elements of the duplication yields a *HIS4*⁺ segregant which has retained the duplication and the intervening plasmid sequences. Thus, a large number of mitotic recombinants can be scored as gene convertants or reciprocal recombinants by colony hybridization using plasmid DNA as a probe¹⁶. Using strains carrying duplications of the *his4* region, we have shown that gene conversion, not associated with reciprocal recombination, is the major event giving rise to *HIS4*⁺ segregants in mitosis. Our studies of these strains also show that the *rad52-1* mutation preferentially decreases mitotic gene conversion.

Construction of the duplication strains

Yeast strains duplicated for the *his4* gene were constructed by transformation¹⁵. The donor DNA was plasmid YIP300 which contains ~24-kb *Bam*HI fragment of yeast DNA inserted into the *Bam*HI site of the vector pBR313¹⁷. The *HIS4*⁺ gene is located near the middle of this *Bam*HI fragment. The recipient was a haploid yeast strain containing two *his4C* mutations (*his4-864* and *his4-1176*) and the *leu2-3* mutation on chromosome III (Fig. 1). Transformation of this stable His⁻ strain to *HIS4*⁺ occurs by the integration of the YIP300 plasmid by homologous recombination. Integration of this plasmid creates a duplication of the *his4* region in one of two possible orientations. The orientation of the *HIS4*⁺, pBR313 and *his4*⁻ sequences with respect to *leu2* depends on the site of integration

relative to the *his4C* mutations (Fig. 2). The *HIS4*⁺ transformants in either orientation are unstable—this instability results from mitotic recombination between elements of the duplication that give rise to *his4*⁻ segregants, and was also observed in yeast strains carrying a duplication of the *leu2* region created by transformation¹⁵.

We devised a positive selection for recombination events in the duplicated region using strains in which each of the two copies of the *his4* gene carry different mutations that can recombine to yield a *HIS4*⁺ segregant. These strains were constructed by crossing individual *HIS4*⁺ *leu2*⁻ transformants with a *LEU2*⁺ strain carrying two mutations in the *his4A* region: *his4-260* and *his4-39*. A His⁻ duplication strain with the structure *his4A*⁻ pBR313 *his4C*⁻ (*his4-260*, *his4-39*, pBR313, *his4-1176*, *his4-864*) can arise from this cross by meiotic recombination event (Fig. 3). Crosses of individual transformants with a *his4A*⁻ *LEU2*⁺ strain give rise to recombinant His⁻ duplication strains of two orientations (Fig. 3). Both orientation I (*his4A*⁻ pBR313 *his4C*⁻ *leu2*⁻) and orientation II (*his4C*⁻ pBR313 *his4A*⁻ *LEU2*⁺) frequently give rise to *HIS4*⁺ segregants by mitotic recombination events.

When the *HIS4*⁺ transformants are crossed by the *his4A*⁻ strain, one parent has the standard arrangement on chromosome III and the other has an insertion of pBR313 flanked by a direct duplication of *his4*. Tetrad analysis of these crosses indicates that neither the duplication nor the pBR313 sequences affect meiotic recombination in the *his4-leu2* interval. Analysis of 101 tetrads yielded a map distance between *his4* and *leu2* of 16.8 centimorgans, which is similar to the *his4-leu2*

Fig. 3 Formation of *his4*⁻ duplication strains of orientations I and II. Transformation with YIP300 yielded duplications of the *his4* alleles in two orientations (Fig. 2). Individual His⁺ transformants of both orientations were crossed by a *LEU2*⁺ strain carrying two mutations in the *his4A* region (*his4-260* and *his4-39*). Four types of His⁻ progeny resulted from this cross; three of these are completely stable and carry a single *his4* region. Colony hybridization using plasmid DNA as the probe shows that they have no plasmid sequences. Stable His⁻ progeny are: (1) a non-recombinant which contains the two *his4A*⁻ mutations (*his4-260* and *his4-39*); (2) a recombinant which contains the two *his4C*⁻ mutations (*his4-1176* and *his4-864*); and (3) a recombinant carrying all four mutations (*his4-260*, *his4-39*, *his4-1176* and *his4-864*). The fourth type is a recombinant which contains a duplication of the *Bam*HI fragment. The formation of this recombinant is shown. One element of the duplication contains the *his4A*⁻ mutations and the other contains the *his4C*⁻ mutations. The orientation of these mutations in the duplication depends on the site of integration of the YIP300 plasmid into the chromosome (see Fig. 2). The *Leu* phenotype of these recombinants is indicative of the orientation of the alleles in the duplication. The His⁻ recombinants carrying a duplication can be easily detected because they frequently give rise to His⁺ segregants by mitotic recombination events occurring between the two *his4* regions, whereas the other His⁻ progeny are completely stable. Colony hybridization using plasmid DNA as a probe shows that the unstable His⁻ progeny retain the pBR313 sequences. Crosses of these strains with wild type (*HIS4*⁺) give rise to recombinant His⁻ progeny containing one or the other component of the duplication. The segregation of each of the components of the duplication in these crosses confirmed the structure in the duplications. *a*, The recombination event giving rise to a *his4*⁻ duplication strain of orientation I; *b*, the recombination event giving rise to a *his4*⁻ duplication strain of orientation II. The thin line represents yeast DNA and the box represents pBR313 sequences.

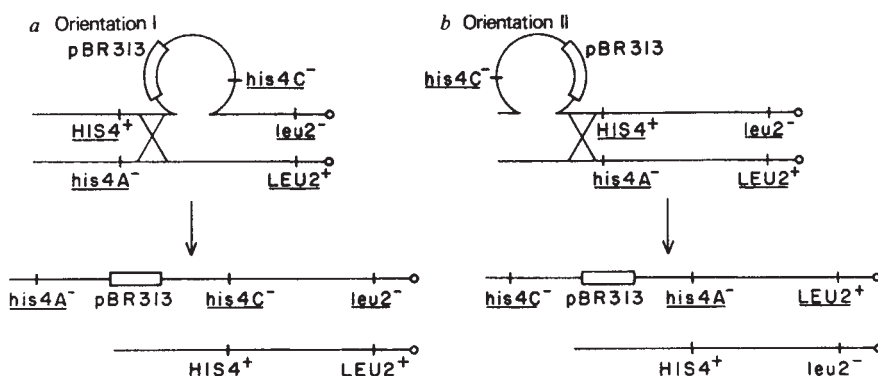


Table 1 Frequency of *HIS4*⁺ formation in duplication strains

Orientation I <i>his4A</i> ⁻ pBR313 <i>his4C</i> ⁻				Orientation II <i>his4C</i> ⁻ pBR313 <i>his4A</i> ⁻			
Strain	Culture no.	<i>HIS4</i> ⁺ frequency per cell ($\times 10^{-5}$)	Mean	Strain	Culture no.	<i>HIS4</i> ⁺ frequency per cell ($\times 10^{-5}$)	Mean
7217-30D <i>RAD</i> ⁺	1	43	21.7 $\pm$ 17.7	7084-48A <i>RAD</i> ⁺	1	16	8.1 $\pm$ 3.7
	2	12			2	6.3	
	3	15			3	8.3	
7313-9B <i>RAD</i> ⁺	1	43		7083-18A <i>RAD</i> ⁺	4	3.7	
	2	5			1	10	
	3	12			2	6.8	
7503-8C <i>rad52-1</i>	1	1.8	2.2 $\pm$ 1.1	96-40B <i>rad52-1</i>	3	5.8	
	2	3.8			1	3.8	
	3	1.2			2	1.9	
	4	1.0			3	2.4	
71-43 <i>rad52-1</i>	5	3.9		95-13D <i>rad52-1</i>	4	2.8	2.5 $\pm$ 0.6
	1	1.3			5	2.1	
	2	3.3			1	2.1	
	3	1.0			2	2.4	

A single colony of the strain to be tested was inoculated into 5 ml of liquid YEPD medium⁵¹ and grown to a concentration of $\sim 10^7$ cells per ml. Cultures were washed, diluted and plated on to five YEPD agar plates for a total cell count and on to five minimal agar plates to estimate the frequency of His⁺ colonies. The plates were incubated at 30 °C for 3 days before counting. The frequency of His⁺ cells was determined by dividing the number of His⁺ cells per ml by the total number of cells per ml.

distance (17.4 centimorgans¹⁸) found in crosses where both parents had the standard arrangement.

Mitotic recombination in haploids and diploids

Mitotic recombination occurs more frequently between two *his4* genes located on a single chromosome III in a haploid than does recombination between *his4* genes located on different chromosome IIIs in a diploid. The spontaneous mitotic recombination behaviour of duplications was studied using haploid strains in which one element of the duplication contained the *his4A*⁻ mutations and the other contained the *his4C*⁻ mutations. The frequency of *HIS4*⁺ formation was measured in these *his4*⁻ duplications when the alleles were in both orientation I and orientation II. *HIS4*⁺ formation was also measured in heteroallelic diploids (*his4A*⁻/*his4C*⁻) of normal chromosome structure. Heteroallelic diploids of mating type *a/a* and α/α were studied in addition to an *a/a* diploid. Formation of *HIS4*⁺ recombinants occurred with a frequency of 0.6×10^{-5} in *a/a* diploids, 0.1×10^{-5} in *a/a* diploids and 0.2×10^{-5} in α/α diploids. The haploid *his4*⁻ duplication strain of orientation I gave rise to prototrophs at a frequency of 21.7×10^{-5} . Orientation II duplications gave rise to prototrophs at a frequency of 8.1×10^{-5} (Table 1). (The difference in frequency between orientations I and II is not statistically significant ($P > 0.90$). Thus, duplications recombine at a frequency which is at least an order of magnitude greater than that measured in diploids.

In meiosis, a diploid containing a duplication on each

homologue (*his4C*⁻ pBR313 *his4C*⁻/*his4A*⁻ pBR313 *his4C*⁻) yields His⁺ recombinants in 1.6% of the tetrads (2/123). Our finding that the frequency of recombination in mitosis is rare compared with that in meiosis agrees with all previous studies on recombination in yeast^{4,7-11}.

Gene conversion is the major recombination event

To study the mitotic recombination events which occur between elements of a duplication, the *HIS4*⁺ recombinants arising from haploid duplication strains were analysed by colony hybridization using pBR313 DNA as the probe. The presence or absence of pBR313 is indicative of the event which gave rise to the *HIS4*⁺ recombinant. Gene conversion unassociated with reciprocal recombination will give a *HIS4*⁺ recombinant which retains the two copies of the *his4* region and the pBR313 sequences (pBR313⁺). A *HIS4*⁺ recombinant with a single copy of the *his4* region and no pBR313 sequences (pBR313⁻) can be generated by two types of event. The first is a simple reciprocal recombination event between the mutant alleles (see Fig. 4). Alternatively, gene conversion of one of the His⁻ alleles to His⁺, followed by reciprocal recombination will give rise to a His⁺ pBR313⁻ recombinant. These two events cannot be distinguished from each other, but as both involve reciprocal exchange they will be classified together as reciprocal recombination.

Analysis of *HIS4*⁺ recombinants arising from duplications in orientation I showed that 15 (12%) had lost the pBR313

Table 2 Frequency of gene conversion and reciprocal recombination among *HIS4*⁺ recombinants

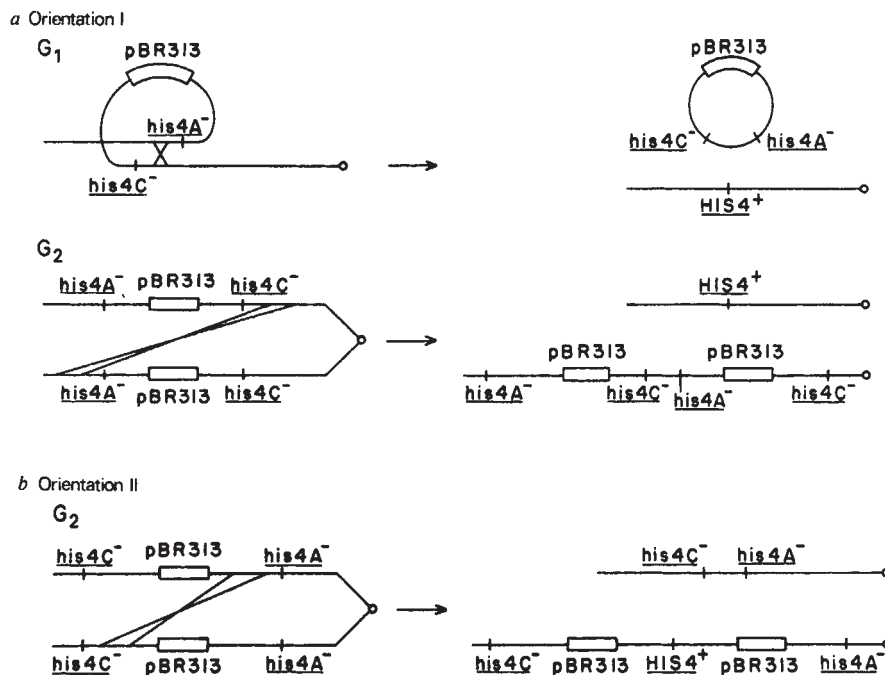
Strain		Colony hybridization				Mean frequency gene conversion ($\times 10^{-5}$)	Mean frequency reciprocal recombination ($\times 10^{-5}$)
		Mean frequency recombination ($\times 10^{-5}$)	No. of colonies tested	% His ⁺ pBR313 ⁺	% His ⁺ pBR313 ⁻		
Orientation I 7217-30D 7503-8C	Rad ⁺	23.3	127	88	12	20.5	2.8
	<i>rad52-1</i>	2.3	192	3	97	0.07	2.2
Orientation II 7084-48A 96-40B	Rad ⁺	8.6	199	97*	3	6.2	2.3
	<i>rad52-1</i>	2.6	146	59†	41	0.18	2.4

Frequency of recombination was determined by dividing the number of prototrophs per ml by number of cells per ml. Mean frequency of recombination is the arithmetic average of frequencies shown in Table 1. Mean frequency of gene conversion was calculated by multiplying % gene conversion, as determined by colony hybridization and Southern analysis, by mean frequency recombination. Mean frequency of reciprocal recombination was calculated by multiplying % reciprocal recombination, as determined by colony hybridization and Southern analysis, by mean frequency recombination.

* Southern hybridization analysis of eight independent His⁺ recombinants showed that 1/4 were triplications and 3/4 were duplications of the *his4* region (see Fig. 5).

† Southern hybridization analysis of eight independent His⁺ recombinants showed that 7/8 were triplications and 1/8 were duplications of the *his4* region (see Fig. 5).

Fig. 4 Formation of *HIS4*⁺ prototrophs by reciprocal recombination. *a*, Reciprocal recombination in orientation I. In *G*₁, before DNA replication, an intra-strand event will yield a *HIS4*⁺ recombinant with a single copy of the *his4* region, and a circular molecule carrying both *his4* mutations and pBR313. In *G*₂, after DNA replication, an inter-strand event will yield a *HIS4*⁺ recombinant with a single copy of the *his4* region, and a *his4*⁻ recombinant with three copies of the *his4* region and two of pBR313. *b*, Reciprocal recombination in orientation II. In *G*₂, an inter-strand event will yield a *HIS4*⁺ recombinant with three copies of the *his4* region and two copies of pBR313. The *his4*⁻ recombinant has a single copy of the *his4* region. In *G*₁, a simple reciprocal recombination event cannot yield a *HIS4*⁺ recombinant. The thin line represents yeast DNA and a box represents pBR313 sequences.



sequences and 112 (88%) had retained them (Table 2). Southern analysis of total yeast DNA using the *Bam*HI fragment of *his4* DNA as a probe showed that the *His*⁺ pBR313⁻ recombinants have a single copy of the *his4* region. This analysis indicates that the recombinants which lose the pBR313 sequences are the result of reciprocal recombination. Confirmation that the major class of recombinants, *His*⁺ pBR313⁺, arose by gene conversion comes from two lines of evidence. First, Southern analysis using the *Bam*HI fragment of *his4* DNA as a probe showed that these recombinants retain two copies of the *his4* region. Second, when any individual *His*⁺ pBR313⁺ recombinant was crossed by an untransformed *HIS4*⁺ strain, either *his4A*⁻ or *his4C*⁻ progeny were recovered. This result indicates that in orientation I most recombinants result from gene conversion.

Most *HIS4*⁺ recombinants which were produced by *his4*⁻ duplications in orientation II were also due to gene conversion. There are three types of *HIS4*⁺ recombinants which result from this orientation. The first type is one which retains the two copies of the *his4* region and one copy of the pBR313 sequences—this type of *HIS4*⁺ recombinant results from a gene conversion event unassociated with reciprocal exchange. The second type is a *HIS4*⁺ recombinant which carries a triplication of the *his4* region and two copies of the pBR313 sequences. This type can be generated by an uneven reciprocal recombination event between sister chromatids (Fig. 4). The third type has a single copy of the *his4* region and no plasmid sequences. This type could result from the conversion of one of the mutant alleles to *His*⁺, followed by a reciprocal recombination event, or alternatively from triple crossovers. Both types 2 and 3 are classified as reciprocal recombinants because their formation involved a reciprocal exchange.

Analysis of 199 *HIS4*⁺ recombinants arising from a strain carrying the duplication in orientation II showed that 193 (97%) had retained pBR313 and 6 (3%) had lost pBR313 (Table 2). To determine which fraction of the *His*⁺ pBR313⁺ recombinants is due to gene conversion, Southern analysis using plasmid DNA as a probe (Fig. 5) was performed on eight independent *His*⁺ pBR313⁺ recombinants. This experiment showed that six of the eight recombinants had two copies of the *his4* region and two had three copies. Assuming that these independent isolates are representative of the whole population, we conclude that ~25% of the *HIS4*⁺ recombinants from orientation II strains are due to reciprocal recombination and 75% are due to gene conversion.

Our finding that most of the *HIS4*⁺ recombinants from the

duplication strains are gene convertants agrees with previous studies on recombination in yeast. Intragenic recombination both in mitosis and meiosis is usually a result of gene conversion rather than reciprocal recombination^{19,20}. It has been shown that up to half of the meiotic conversion events are associated with reciprocal recombination⁴. In contrast, the major mitotic recombination event observed in duplications is gene conversion, unassociated with reciprocal exchange. Our evidence for this conclusion is based on the retention of the duplication and plasmid sequences in most *HIS4*⁺ recombinants. This result agrees with studies of mitotic recombination in diploids^{7,8}, and suggests that mitotic gene conversion may proceed by a mechanism which is different from that in meiosis.

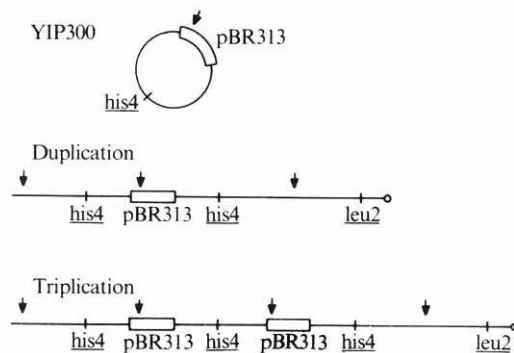
These observations on duplications of the *his4* locus apply to other regions of the yeast genome. Duplications of the *leu2* gene, with the structure *leu2-101 colE1 leu2-3 leu2-112*, behave identically to the *his4* duplications. About 95% of the *LEU2*⁺ recombinants are gene convertants. In a duplication with the structure *LEU2*⁺ *ColE1 leu2-3 leu2-112*, 17 *Leu*⁻ segregants were generated by a recombination event which separated *leu2-3* from *leu2-112*. All these segregants retained the vector sequences (eight were *leu2-112 ColE1 leu2-3 leu2-112* and nine were *leu2-3 ColE1 leu2-3 leu2-112*) (C. Styles and G. R. Fink, unpublished data). Thus, a high frequency of gene conversion occurs in prototrophic or auxotrophic recombinants as long as the event is intragenic.

Previous work²¹⁻²³ has shown that spontaneous and induced intragenic mitotic recombination can occur before DNA synthesis, at the two-strand stage. The reciprocal recombination which we observe in the duplication strains suggests that mitotic recombination is not restricted to one stage of the cell cycle. The fact that duplication strains in orientation II give rise to prototrophs which carry a triplication of the *his4* region (Fig. 4) argues that, in a haploid, mitotic recombination can occur after DNA synthesis. This is the first evidence of sister-strand exchange in chromosomal regions outside the rDNA cistrons^{24,25}. It is unclear what proportion of the mitotic recombination events occur at this stage, because the other classes of recombinants could be produced either before DNA replication by an intra-strand recombination event or after DNA synthesis by an inter-strand event.

Gene conversion is decreased by *rad52-1*

The *rad52-1* mutation has been reported to abolish meiotic recombination and to reduce mitotic recombination¹²⁻¹⁴. We

Fig. 5 Quantitation of *his4* regions. The diagram shows the configuration of the plasmid YIP300, a strain carrying a duplication of the *his4* region and a strain carrying a triplication of this region. The thin line represents yeast DNA; the box represents pBR313 sequences and arrows represent *Sma*I endonuclease sites. Also shown is an autoradiogram of the hybridization profile of *Sma*I-digested DNA from a duplication; b, a triplication and c, YIP300 using labelled plasmid as a probe. The two *Sma*I fragments carrying pBR313 sequences in the duplication migrate together on an agarose gel. These two fragments are found in a triplication that has, in addition, a smaller *Sma*I fragment carrying pBR313 sequences which is the same size as the YIP300 plasmid. Nuclear DNA was isolated from the yeast strains by the method of Cryer *et al.*⁴⁶. Plasmid YIP300 was isolated as described by Hirt⁴⁷. After digestion with *Sma*I the fragments were separated by agarose gel electrophoresis on a horizontal 0.5% agarose slab gel run at 100 V for 18 h. DNA was transferred to nitrocellulose filters⁴⁸ which were hybridized to a DNA probe ³²P-labelled by nick translation⁴⁹. The probe was pBR322, a plasmid derived from pBR313⁵⁰.



found that the *rad52-1* mutation lowers the frequency of *HIS4*⁺ formation for *his4*⁺ duplications of both orientations I and II. Further analysis of the *HIS4*⁺ recombinants produced in a *rad52-1* background indicate that gene conversion is affected preferentially. For orientation I, *rad52-1* lowered the frequency of *HIS4*⁺ formation ~10-fold (Table 1). When *HIS4*⁺ recombinants were examined by colony hybridization using plasmid DNA as a probe, 186 (97%) had lost the pBR313 sequences (Table 2). In comparison, only 12% of the *HIS4*⁺ recombinants produced by the *RAD52*⁺ strains in orientation I had lost pBR313. The loss of the plasmid sequences indicates that a reciprocal recombination event had occurred (Fig. 4). When the overall frequency of *HIS4*⁺ formation in orientation I strains is separated into reciprocal recombination and gene conversion components, the component representing the reciprocal recombination frequency is essentially the same in the *rad52-1* and *RAD52*⁺ backgrounds (Table 2). Thus, the 10-fold decrease in *HIS4*⁺ formation in *rad52-1* strains is due to a greater than 200-fold decrease in gene conversion events.

The *rad52-1* mutation lowered the frequency of *HIS4*⁺ formation in duplication strains of orientation II threefold. Colony hybridization using plasmid DNA as the probe showed that 145 (59%) of the *HIS4*⁺ recombinants had retained pBR313 and 101 (41%) had lost this sequence (Table 2). The proportion of *His*⁺ pBR313⁺ recombinants which were due to reciprocal recombination was determined by Southern hybridization analysis of whole yeast DNA from eight independent isolates (Fig. 5). This analysis showed that seven of the eight *His*⁺ pBR313⁺ recombinants carried a triplication of the *his4* region, and therefore were a result of a reciprocal recombination event. The other *His*⁺ pBR313⁺ recombinant carried a duplication of the *his4* region. As the eight strains are independent isolates, we assume that they are representative of the entire *His*⁺ pBR313⁺ population. On this assumption, 88% of the *His*⁺ pBR313⁺ and all the *His*⁺ pBR313⁻ recombinants, or 93% of the total *HIS4*⁺ recombinants arising in a *rad52-1*, orientation II background are due to reciprocal recombination. When the overall frequency of *HIS4*⁺ formation is separated into reciprocal recombination and gene conversion components, the component representing the reciprocal recombination frequency is the same as that of the *Rad52*⁺, orientation II strain, and gene conversion has been decreased ~30-fold (Table 2).

There is a difference in the extent to which *rad52* decreases the frequency of gene conversion in the strains of orientations I and II (Table 2). Others have reported that the effect of *rad52* on mitotic recombination varies from 6- to 200-fold depending on the genetic interval studied¹²⁻¹⁴. The differences we observed may be due to the orientation of the *his4* alleles or to differences in the genetic backgrounds of the strains used here.

Studies on the effect of the *rad52-1* mutation in diploid strains have shown that this allele affects mitosis and meiosis differently¹²⁻¹⁴. Meiotic recombination is completely abolished and mitotic recombination decreased. We have shown that in strains carrying the *rad52-1* mutation the frequency of gene conversion not associated with reciprocal exchange is decreased dramatically, whereas the frequency of reciprocal recombinants is not affected (Table 2). The *His*⁺ prototrophs classed as reciprocal recombinants could be generated by gene conversion followed by reciprocal exchange. If this association of gene conversion with reciprocal recombination is frequent, then *rad52* only affects mitotic gene conversion that is not associated with reciprocal exchange.

Discussion

We have shown that the major mitotic recombination event observed in duplications is gene conversion unassociated with reciprocal exchange. This result is consistent with the results of previous studies on mitotic recombination between single-copy genes in heteroallelic diploids^{7,8}. In addition, we observe that the *rad52-1* mutation, which decreases mitotic exchange in diploids^{12,14}, preferentially decreases gene conversion in our system. These findings, taken together with earlier studies, suggest that gene conversion and reciprocal recombination occur by different pathways in mitosis.

Gene conversion between repeated DNA sequences may be involved in biologically important phenomena in yeast. For example, mating type interconversion is a complex event which seems to have some features in common with gene conversion within gene duplications^{26,27}. Mating type in yeast is controlled by two alleles of the *MAT* locus, *MATa* and *MATα*. Homothallic strains switch from one mating type to the other at a high frequency^{28,29}. This switching of mating type requires two other loci, *HML* and *HMR*³⁰⁻³². *HML* and *HMR* are unexpressed copies of the α and a information which can donate this information to the *MAT* locus^{33,34}. The replacement of *MAT* information by *HML* or *HMR* does not lead to a change at the donor locus. This aspect of homothallic switching is similar to gene conversion within duplications. One copy of the duplication acts as a donor of genetic information and the other as recipient. The donor sequences are not altered during the event. In contrast to mitotic gene conversion, mating type interconversion is a directed event; a information replaces *MATa* and α information replaces *MATα*^{33,34}. Studies of cloned mating type genes show that the *MAT*, *HML* and *HMR* loci are direct repeats of common DNA sequences on chromosome III^{35,36}. Thus, mating type loci are like the non-tandem direct repeats of the *his4* gene which we have studied. Unlike the *his4* gene duplications, where the two regions are completely homologous, each mating type locus contains one of two non-homologous

sequences (an 850-bp α -specific sequence or a 700-bp α -specific sequence) flanked by sequences homologous to all three loci³⁶. In spite of the differences between mating type interconversion and mitotic gene conversion, both involve the transfer of genetic information without rearrangement of the donor sequences. Moreover, the suggestion that the two processes occur by a similar event is supported by the finding that homothallic strains carrying the *rad52-1* mutation are defective in mating type interconversion¹³.

Sequence homogeneity between highly repeated DNA sequences is thought to be maintained by mitotic recombination. In yeast, rDNA is composed of ~100 repeats of a 9-kb unit per haploid genome³⁷. Genetic and biochemical evidence suggests that the rDNA repeat units are tandemly arrayed, and within any individual strain these repeat units are homogeneous³⁸⁻⁴¹. Unequal sister-strand reciprocal recombination has been shown to occur in the rDNA cistrons in both mitosis and meiosis of

yeast^{24,25}. Szostak and Wu²⁵ propose that unequal sister-strand exchange occurs at a high enough frequency in mitotic cells to account for the homogeneity. Meiotic analysis of a duplication of the *leu2* gene in yeast has led Klein and Petes⁴² to suggest that gene conversion is a more likely mechanism for rDNA rectification. Our data indicate that mitotic gene conversion is far more frequent than unequal sister-strand exchange and therefore more likely to be responsible for sequence homogeneity. These conversion events could be responsible for the sequence homogeneity found in other types of repeated genes in eukaryotes, for example, the histone⁴³ and globin genes^{44,45}.

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Chromatin structure of endogenous retroviral genes and activation by an inhibitor of DNA methylation

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The transcriptionally active ev-3 and inactive ev-1 endogenous retrovirus loci in chick cells differ in that ev-3 is undermethylated, preferentially sensitive to DNase I digestion, and contains nuclease hypersensitive sites in each of its two long terminal repeats. Transient exposure of cells to 5-azacytidine, a cytosine analogue which cannot be methylated at the 5 position, results in the hypomethylation and transcriptional activation of ev-1, as well as the acquisition of at least one nuclease-hypersensitive site within the chromosomal domain of ev-1.

ANALYSES from several laboratories have shown that expressed genes are packaged into an altered chromatin structure, as revealed by preferential DNase I digestion¹⁻⁴. These active regions are usually undermethylated at CpG dinucleotides⁵⁻¹². In many cases, gene activity is also correlated with a DNase I-hypersensitive site, which represents a very local alteration of chromatin that is associated with certain specific DNA sequences. These 'hypersensitive' regions are detected by the ability of DNase I to introduce double-stranded cuts at such

specific DNA sequences¹³. Thus, when nuclei are mildly digested with DNase I and the DNA isolated and redigested with restriction endonucleases, a sharp 'sub-band' appears on 'Southern' blotting. One end of this fragment is defined by the DNase I-generated double-stranded cuts in chromosomal DNA, and the other end by a neighbouring restriction site. Although many of the hypersensitive sites described thus far are very near the 5' border of transcriptionally active genes, others have been located in adjacent non-coding regions^{14,15}.

The notion that hypersensitive sites may reflect important events in gene regulation is further supported by the finding that a change in location of these sites accompanies haemoglobin switching in the developing chicken embryo^{14,16}. Recently, we have shown that in precursor erythroblasts obtained from 22-h chick blastoderms, the globin genes are not transcribed; they are methylated, DNase I resistant and contain no hypersensitive sites. However, because all these parameters of gene activity become expressed coordinately (within the temporal resolution of our experiments) in progeny erythroblasts, it is not clear which, if any, of these structural features of active chromatin are primary and which secondary. We thus sought a system where we could experimentally manipulate such parameters.

In attempting to study the relationships between transcription, DNase I sensitivity, CpG methylation and hypersensitive sites, we have investigated these aspects of the endogenous avian retroviral genomes. Uninfected chicken cells contain one or more complete or nearly complete copies of genetically transmitted endogenous viruses, the prototype of which is called RAV-O. As described by Astrin¹⁷, most chickens contain at least one such genetic locus, *ev-1*, identifiable on 'Southern' blots as a 10.5-kilobase (kb) *Sst*I restriction fragment^{18,19}. Although *ev-1* contains structural sequences nearly identical to RAV-O, it is basically silent in terms of expression²⁰. Astrin and co-workers²¹ have described at least 10 other avian genetic loci that contain structural genes for endogenous retroviruses, all of which are identifiable as specific *Sst*I fragments. Of particular interest is *ev-3*, a deleted RAV-O locus which serves as the template for 31S and 22S viral RNA transcripts in uninfected chicken cells^{20,22}. Although *ev-3*-containing cells do not produce either infectious virus or virus particles, they do contain a 120,000-molecular weight *ev-3*-coded polyprotein, P120^G, which represents an apparently non-functional, internally deleted form of the precursor to reverse transcriptase found in retrovirus infected cells^{23,24}. The specific region of the *ev-3* deletion and detailed restriction maps of the various *ev* loci have been described recently^{18,19,21,23,24}.

The present experiments demonstrate that, in contrast to *ev-1*, the conformation of *ev-3* in chromatin results in sensitivity of this locus to digestion by DNase I and site-specific double-stranded cleavage at both the 5' and 3' ends of *ev-3* (in long terminal repeat (LTR) sequence) by both DNase I and staphylococcal nuclease. In addition, and again in contrast to *ev-1*, several regions of *ev-3* are undermethylated at cytosine residues at CpG dinucleotides. That undermethylation of at least some of these sites is important in permitting expression of the endogenous retroviral genome is strongly suggested by the transcriptional activation of an *ev-1* locus by 5-azacytidine, a cytosine analogue which, due to a nitrogen substitution in the 5 position, cannot be methylated at this site²⁵. The activation of *ev-1* by 5-azacytidine is also correlated with the generation of a

hypersensitive site within the chromosomal domain of this previously inactive retroviral genome. These results suggest that, at least for the *ev-1* locus, undermethylation of at least part of the *ev-1* locus results in the subsequent chromosomal activation of this genome as revealed by DNase sensitivity, transcription and synthesis of viral proteins.

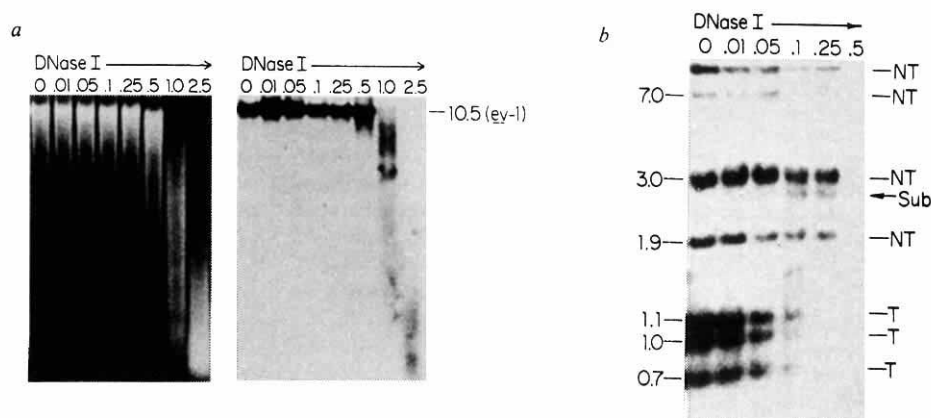
Preferential nuclease sensitivity of *ev-3*

Nuclei were isolated from red blood cells of 14-day chicken embryos characterized genotypically as either *ev-1* or *ev-1/ev-3*. As previously reported^{4,11,15}, although such erythrocytes are no longer transcriptionally active, the globin genes previously expressed in these cells are preferentially sensitive to DNase I. The respective nuclear preparations were digested with very low levels of DNase I or staphylococcal nuclease^{4,13,15} and the DNA was purified, restricted with *Sst*I, run on neutral 1% agarose gels, 'Southern' blotted and hybridized to ³²P-RAV-O cDNA. (In all cases, identical results were obtained using cloned RAV-O sequence containing DNA as probe; however, we prefer to use the cDNA because it is usually higher in specific activity and gives much less background on blots.) Before restriction, most of this DNA had an average size of 15–20-kilobase pairs (kbp).

Figure 1 shows the results of DNase I digestion of nuclei from *ev-1* red blood cells. As predicted from known restriction maps of *ev-1*^{18,19,21}, one major band is apparent. However, neither preferential digestion of *ev-1* compared with bulk DNA, nor sub-bands indicative of site-specific double-stranded cuts within this locus are observed (Fig. 1a). Digestions with several other enzymes also fail to demonstrate sub-bands, indicating the lack of specific cuts in either the 5'- or 3'-flanking regions of this gene (not shown). As controls for the preferential digestion of active genes and generation of specific sub-bands in these DNA samples, restriction bands containing the α -globin genes once transcribed in these cells are no longer detectable in the sample digested with 0.25 $\mu\text{g ml}^{-1}$ DNase I, and several samples show a specific sub-band containing globin flanking sequences (Fig. 1b).

Figure 2a reveals the results of a similar analysis performed with nuclei from cells containing both *ev-1* and *ev-3*. In addition to confirming the relative 'insensitivity' of *ev-1* to DNase I digestion, this figure demonstrates that *ev-3* is much more sensitive to DNase I than is *ev-1*. Thus, the chromosomal conformation of *ev-3* is similar to many transcriptionally active genes assayed by this technique. The kinetic analysis of the preferential digestion of the 6.5-kbp *Sst*I restriction fragment containing *ev-3* also reveals a 5.8-kbp sub-band, indicating a site-specific double-stranded cut introduced into the chromosomal DNA in or around this locus. A similar sub-band is detected when nuclei from *ev-1/ev-3* red cells are digested with increasing concentrations of staphylococcal nuclease (Fig. 2b), further indicating that a specific region of this endogenous viral locus is uniquely cut in the intact chromosome. Note,

Fig. 1 a, Resistance of *ev-1* to low level DNase I digestion. Red blood cell nuclei from 14-day chicken embryos of *ev-1* genotype were incubated with increasing amounts of DNase I at a DNA concentration of 1 mg ml^{-1} for 10 min at 37°C. DNA was isolated, restricted with *Sst*I, electrophoresed on a neutral 1% agarose horizontal slab gel, stained with ethidium bromide, blotted on to a nitrocellulose filter and hybridized to ³²P-RAV-O cDNA. The dominant RAV-O related band in the *Sst*I digest is 10.5 kbp, corresponding to *ev-1*. Numbers at the top of each lane refer to concentration ($\mu\text{g ml}^{-1}$) of DNase I. b, Sensitivity of globin DNA to DNase I. The same DNA as shown in a was digested with *Bam* and *Eco*RI and hybridized to a genomic clone (α_2) containing transcribed (T) and non-transcribed (NT) regions of the chicken α -globin domain (the sub-band is generated from a hypersensitive site in a non-transcribed region of the α locus; see ref. 11 for details). The NT regions show a marked DNase sensitivity that has previously been shown to depend on HMG proteins, while the T regions show an 'intermediate' level of sensitivity that is not related to HMG binding. The *ev-1* locus in a is classified as being DNase 'resistant'.



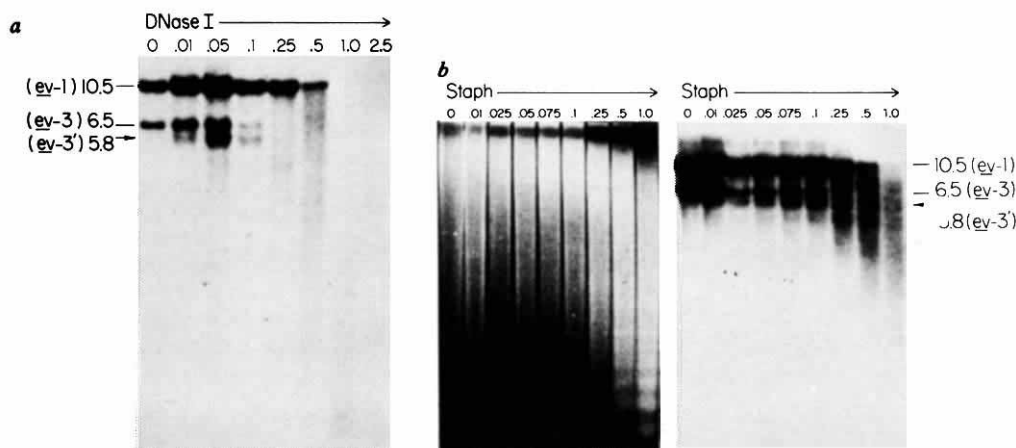


Fig. 2 Preferential sensitivity of *ev-3* to DNase I and the generation of *ev-3*-specific sub-bands by DNase I and staphylococcal nuclease. *a*, *ev-1/ev-3* DNA was prepared and analysed after restriction with *Sst*I as described for *ev-1* DNA in Fig. 1. Two dominant RAV-O related bands in the *Sst*I digest are the 10.5-kbp *ev-1* and the 6.5-kbp *ev-3*. In addition to the preferential sensitivity of *ev-3* to DNase I, a 5.8-kbp sub-band is revealed. *b*, DNA from staphylococcal nuclease-treated red blood cell nuclei from 14-day chicken embryos of *ev-1/ev-3* genotype was prepared and analysed as above.

however, that the dramatic disappearance of *ev-3 vis-à-vis ev-1* seen in the DNase I digest (Fig. 2a) is not observed with staphylococcal nuclease digestion (Fig. 2b).

Previously, we reported the presence of RAV-O-related RNA and proteins in cells isolated from embryos of a particular flock of P120^{Gs}-negative, chick helper factor (*chf*) negative (*gs⁻chf⁻*), uninfected chickens²⁶. Using DNase I and solution hybridization we observed that these cells contained one 'active' and one 'inactive' retroviral genome. However, restriction mapping was not performed and the *ev* loci were not identified. Given the recent description by Astrin and co-workers²¹ of multiple *ev* loci associated with the *gs⁻chf⁻* phenotype, we re-analysed cells from this flock and observed that they contain both *ev-1* and another 'phenotypically negative' *ev* locus, *ev-8*. Whereas *ev-8* is DNase I sensitive in these cells, the *ev-1* locus is resistant to DNase I, consistent with the above results. (A detailed analysis of these cells will be presented elsewhere.)

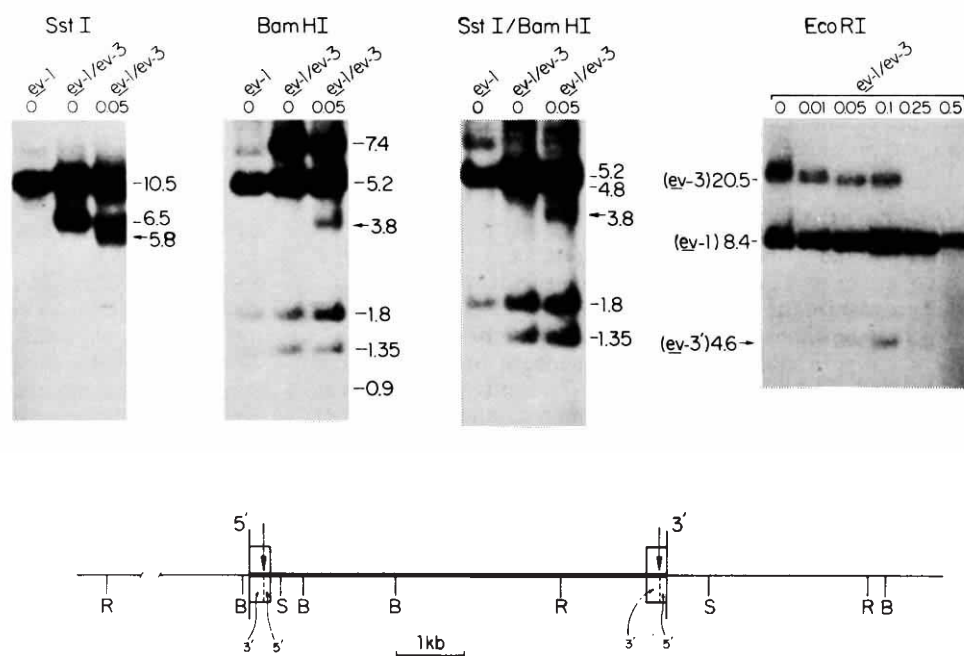
Mapping the hypersensitive site of *ev-3*

In an attempt to understand better the relationship between hypersensitive sites and gene activity, we mapped the region of *ev-3* uniquely cut by DNase I and staphylococcal nuclease. Although all the mapping data presented here were generated from experiments with DNA from DNase I-digested nuclei, the same results are obtained with DNA samples from staphylococcal

nuclease-treated nuclei (not shown). Figure 3 shows the results of this analysis and a restriction map of *ev-3*. As indicated above (and in Fig. 3), a sub-band of 5.8 kbp is generated when the DNase I-treated samples are digested with *Sst*I. Given the restriction map of *ev-3*, the hypersensitive site observed with *Sst*I must be located either 700 bases 3' to the *Sst* site in the 5' end of the locus or within the LTR located on the 3' side of *ev-3*. When the same sample was digested with *Bam*HI, a sub-band of 3.8 kbp was observed, indicating that the sub-band must be generated from a site within the 3' LTR. *Sst*I/*Bam*HI double digestion (Fig. 3) also shows a 3.8-kbp sub-band, providing further support for this conclusion. Given the location of *Bam* sites in the 5' end of the locus, this sub-band could only be generated by a DNase I-generated double-stranded cut in the 3' LTR.

As the LTR is repeated at both ends of *ev-3*, we wondered if a similar hypersensitive site was located in the LTR on the 5' side of the locus. Neither *Sst*I nor *Bam* digestion would reveal such a site, because both cleave within the locus, 3' to the 5' LTR. We therefore hybridized an *Eco*RI-digested DNase series from *ev-1/ev-3* cells with a ³²P-nick-translated DNA fragment containing sequences specific to the 'gag' gene located to the right of the 5' LTR. (The fragment was isolated from a pBR subclone provided by Drs W. DeLorbe and J.M. Bishop and their colleagues.) This probe detected the two expected *Eco*RI-generated restriction fragments in the undigested sample

Fig. 3 Mapping the specific DNase I-hypersensitive sites in *ev-3*. The restriction map of *ev-3* is derived primarily from our own results, and the work of Hughes *et al.*¹⁹. Undigested DNA from *ev-1* and *ev-1/ev-3* erythrocytes, and DNA from *ev-1/ev-3* nuclei digested with 0.05 µg ml⁻¹ DNase I were restricted with either *Sst*I or *Bam*HI or doubly digested with *Sst*I and *Bam*HI, and analysed as described in the previous figure legends. In addition, the DNase I-digested *ev-1/ev-3* DNA 'series' presented in Fig. 2 was digested with *Eco*RI and analysed as above except that rather than using ³²P-RAV-O cDNA, we used a ³²P-nick-translated DNA fragment containing sequences specific to the *gag* gene located to the right of the 5' LTR. In the *Eco*RI blot, the 20.5-kbp fragment is *ev-3*-specific, whereas the 8.4-kbp fragment is *ev-1* specific. (In mapping the location of the nuclease-generated sub-bands with *Eco*RI, we have presented a kinetic analysis for optimal visualization of the *ev-3* sub-bands.)



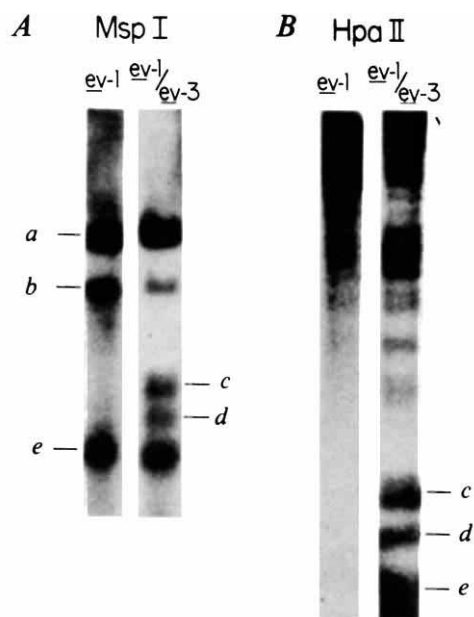


Fig. 4 Undermethylation of *ev-3* relative to *ev-1*. DNA from *ev-1* and *ev-1/ev-3* erythrocytes was purified from nuclear preparations, digested with various restriction enzymes, electrophoresed on neutral, 1.4% agarose horizontal slab gels, blotted on to nitrocellulose and hybridized to ³²P-RAV-O cDNA. **A**, *MspI* digestion of *ev-1* and *ev-3* DNA. **B**, *HpaII* digestion of the same samples. Note the generation by *HpaII* of fragments *c*, *d* and *e* in the *ev-1/ev-3* cells, but not in *ev-1* cells.

(20.5 kbp containing the 5' 5.0 kbp of *ev-3* and 15.5 kbp of 5' flanking sequences, and an 8.4-kbp *ev-1* specific fragment), but revealed a 4.6-kbp sub-band in the DNase I-digested samples (Fig. 3). As we have been unable to detect any retrovirus-related sub-bands in DNA from DNase-treated *ev-1* nuclei using a variety of restriction enzymes and specific probes (Fig. 1 and other data not shown), the 4.6-kbp sub-band is probably generated from a hypersensitive site that maps in the 5' LTR of *ev-3*. This has been confirmed using several other probes and restriction enzymes, and we are now attempting to determine the exact sequence within the LTR that is cut by DNase I.

Thus, although no hypersensitive sites are observed in the *ev-1* chromosomal domain, at least two such sites are apparent in *ev-3*, both located within regions of sequence homology (LTRs) on both the 5' and 3' side of the proviral coding regions. These LTR regions are of particular interest in terms of control of gene expression, given the sequence homology in this region to a number of putative control elements²⁷ (see discussion) and the ability of homologous regions in Rous sarcoma virus (RSV) to act as promoters *in vitro*²⁸.

Undermethylation of *ev-3*

Figure 4A and B show the results of restricting DNA from red cells of either *ev-1* or *ev-1/ev-3* genotype with *MspI* and *HpaII*, which recognize the sequence CCGG²⁹; however, *MspI* cuts at this site independent of the methylated state of the second C, whereas *HpaII* will not cut this sequence if the second C is methylated. As evident in Fig. 4A, *ev-1/ev-3* DNA contains at least two more *MspI* restriction fragments (labelled *c* and *d*) than *ev-1* DNA. Figure 4B illustrates that although *ev-1* is highly methylated at CCGG sequences, the two *ev-3*-specific *MspI* fragments (*c* and *d*) are not methylated at such residues. In addition, several other regions are relatively undermethylated, given the generation of a broad band (*e*) of fragments with an average size of 300 base pairs (bp). We presume that these fragments come from *ev-3* as they are not found in *HpaII*-digested DNA from *ev-1* birds; however, we cannot formally rule out the possibility that under the influence of *ev-3*, these

fragments now become undermethylated at the *ev-1* locus. We have also observed that whereas the 10.5-kbp *SstI* fragment characteristic of *ev-1* is relatively stable on double digestion of *ev-1* or *ev-1/ev-3* DNA with *SstI* and *HpaII*, the 6.5-kbp *SstI* fragment characteristic of *ev-3* disappears on *SstI/HpaII* double digestion of *ev-1/ev-3* DNA (not shown). Thus, our results demonstrate qualitatively a clear correlation between the expressed *ev-3* locus and undermethylation and an analogous correlation between the unexpressed *ev-1* locus and hypermethylation. Although other investigators have also observed differences in the methylated state of retroviral genomes in various cells³⁰⁻³², correlation of expression and degree of methylation of a particular viral locus in these cells was not possible.

Induction of *ev-1* expression by 5-azacytidine

In an attempt to understand the role of undermethylation in the control of gene activity, we analysed the effect of 5-azacytidine²⁵ on the expression of the inactive *ev-1* locus. A continuous line of chicken lymphocytes containing only *ev-1*, MSB cells^{33,34}, was exposed to the cytosine analogue for 24 h (two or three generations), the medium containing the drug removed, fresh medium added and the cells assayed at various intervals for changes in *ev-1* DNA methylation, RNA accumulation and virus-specific protein synthesis. DNA containing this analogue cannot be methylated at the substituted site²⁵. Moreover, during the 'chase' period most of the analogue is diluted out by replication. Thus, its effect on methylation occurs during the initial treatment, but the undermethylated state is nevertheless propagated to daughter cells independent of the continual presence of the analogue.

Figure 5A shows *MspI* and *HpaII* digestions of DNA from control and 5-azacytidine (AZA)-treated MSB cells. Although *ev-1* *MspI* fragments are generally similar in red blood cells (Fig. 4) and MSB cells (Fig. 5), a small amount of what may be partial and limit restriction fragments is present in *HpaII* digests of MSB [for example, bands *c* and *d* in Fig. 5A (left)], but not erythrocyte DNA when blots containing restricted DNA from both cell types are exposed equivalently. As the MSB cells are routinely subcloned, these limit *HpaII* fragments may reflect the fidelity with which a particular methylation pattern is transmitted to daughter cells during DNA replication.

In contrast to the very small amount of signal observed in the limit restriction fragments from control MSB cells, exposure of these cells to azacytidine results in the generation by *HpaII* of the major *MspI* *e* fragments observed in control DNA. Based on the relative intensities of the *e* band observed in *MspI* and *HpaII* digests (Fig. 5A), within the limits of our assay, ~50% of the *ev-1* *MspI* fragments in these cells are now undermethylated. We presume that this reflects the degree of substitution of 5-azacytidine during the initial treatment period.

To assay for gene expression, we isolated cytoplasmic RNA from control and azacytidine-treated MSB cells, 'dotted' the RNA on to nitrocellulose³⁵ and assayed for the appearance of RAV-O-related sequences by hybridization with ³²P-RAV-O cDNA (Fig. 5B). In these conditions we cannot detect significant signal from control cells, but signal from the AZA-MSB cells is observed as early as 24 h after removal of the analogue. Although the total amount of viral RNA increases between 24 and 48 h post-exposure, virus-specific RNA sequences change little between 48 and 72 h. To quantify *ev-1*-coded RNA sequences, we compared the intensities of the AZA-MSB RNA 'dots' with that from RNA isolated from chick embryo fibroblasts (CEF) infected with RSV, which by solution hybridization analysis contain ~5,000 copies of virus-specific RNA per cell (not shown). As the intensity of the 10 µg AZA-MSB 'dots' is comparable with that of the 0.1-µg RSV-CEF 'dots', we estimate that AZA-MSB cells contain ~50 copies of RAV-O-related RNA per cell. This has also been confirmed by conventional hybridization analysis in solution. Obviously, this assay gives no information regarding the number of cells actually transcribing *ev-1* (see below); however, here we are interested

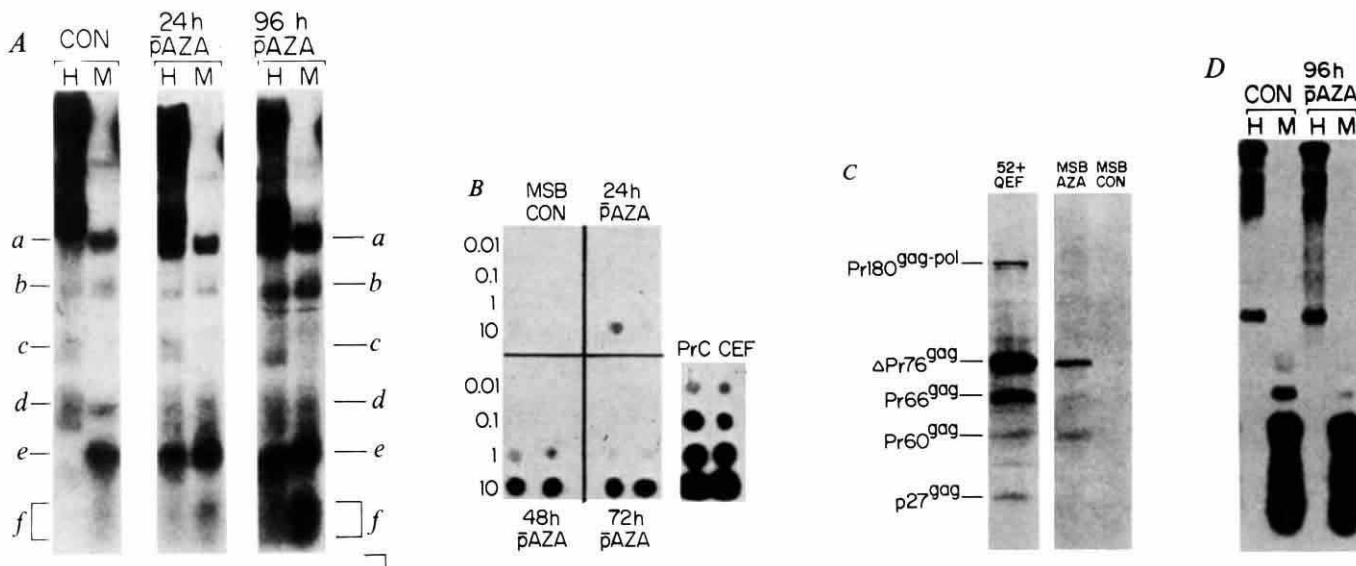


Fig. 5 Induction of *ev-1* expression by 5-azacytidine. MSB cells were grown in media containing 3 μ M 5-azacytidine (AZA) for 24 h, the analogue-containing medium was removed and the cells analysed 24, 48, 72 or 96 h after removal of the drug. **A**, Restriction of DNA from control (CON) and 5-azacytidine-treated (AZA) MSB cells with *HpaII* (H) and *MspI* (M) 24 and 96 h after removal of the analogue, and hybridization to 32 P-RAV-O cDNA. **B**, 'Dot blots' of cytoplasmic RNA isolated from control MSB cells, MSB cells at 24, 48 or 72 h after exposure to 5-azacytidine, and chick embryo fibroblasts (CEF) infected with RSV. 10, 1, 0.1 and 0.01 μ g of each RNA sample were denatured at 65 $^{\circ}$ C, spotted in duplicate on to dry nitrocellulose filter paper (previously wetted with H_2O and soaked in 20 $\times$ SSC for 30 min), dried under a heat-lamp, and baked at 80 $^{\circ}$ C under vacuum for 2 h. The RNA-containing filters were prehybridized and then hybridized to 32 P-RAV-O cDNA. **C**, Analysis of retrovirus proteins in untreated or azacytidine-treated MSB cells. MSB cells were treated with 3 μ M azacytidine for 24 h and allowed to grow for 3 additional days after removal of the drug. Untreated cells from the same culture were grown in parallel. The cells were washed and incubated for 1 h at 37 $^{\circ}$ C with 200 μ Ci 35 S-methionine in methionine-deficient medium. Quail embryo fibroblasts (QEF) productively infected with the wild-type virus 52+ were similarly labelled. Cells were lysed as previously described^{23,24} and treated with 4 μ l of a rabbit antiserum raised against purified retrovirus gag proteins and 80 μ l of fixed *Staphylococcus aureus*. The immune complexes were washed extensively, dissolved in SDS (5%) electrophoresis sample buffer and subjected to electrophoresis on a 12.5% polyacrylamide slab gel; an autoradiograph of the dried gel is shown. The various gag-related polypeptides immunoprecipitated with the antiserum represent the different cleavage proteins generated from the primary precursor, Pr76^{gag}. **D**, Hybridization of a 32 P-nick translated, embryonic β -globin gene containing plasmid to *HpaII* (H)- and *MspI* (M)-digested DNA from control (CON) and 5-azacytidine-treated (AZA) MSB cell populations 96 h after removal of the analogue.

in any transcription of the retroviral locus in the AZA-cells compared with control MSB cells. Interestingly, analysis of viral RNA content in cells containing the active endogenous *ev-3* genome has revealed that such cells also contain ~ 50 copies of RAV-O-related RNA per cell²².

We also investigated the synthesis of viral proteins in the 5-azacytidine-treated cells. MSB cells have been characterized as negative for endogenous retroviral *gag*, *pol* and *env* gene expression³⁶ and, as shown in Fig. 5C, we could not detect any of the viral internal structural proteins (gag) in the control MSB cells. However, Fig. 5C also shows that the gag precursor polyprotein Pr76^{gag} and two of its cleavage intermediates, Pr66 and Pr60, are detectable in MSB cells assayed 72 h after exposure to azacytidine. Of significance, viral particles are also detectable in the supernatant medium from these cells, but they do not contain reverse transcriptase (R. Eisenman and K. Conklin, unpublished observations). The absence of reverse transcriptase actually facilitates our assay, for the particles are thus uninfected, and our results cannot be attributed to re-infection of the AZA-MSB cells by RAV-O. (A detailed characterization of the viral RNA, protein and particles is in preparation.)

As expected from the results of Jones and Taylor²⁵, our results depend on incorporation of the analogue into cellular DNA; concurrent exposure of MSB cells to azacytidine and inhibition of DNA synthesis by cytosine arabinoside or hydroxyurea results in none of the observed changes in DNA methylation or viral RNA accumulation (not shown). We have also observed that 24 h exposure of MSB cells to concentrations of azacytidine $> 7 \mu$ M results in severe toxicity, with only 10–20% of the cells remaining viable 48 h after its removal. At the concentration used in these experiments (3 μ M), a minimum of 50% of the cells are viable when assayed at 48 h post 'wash-out'. We have also observed *ev-1* activation on 8 h exposure of MSB cells to 1 μ M azacytidine and, in these conditions, $> 90\%$ of the cells remain viable. Finally, we have observed similar changes in *ev-1*

DNA methylation, RNA accumulation and protein synthesis on exposure of cultures of *ev-1* containing chick embryo fibroblasts to the analogue (unpublished observations). Thus, azacytidine is effective both in activating whole programmes of differentiation in mouse cells²⁵ (see discussion) and in activating a particular gene in chicken cells, in a continuous line and in primary fibroblasts.

In contrast to the 5-azacytidine-induced undermethylation of regions of *ev-1*, we have observed no significant changes in the methylated state of five different α - and β -globin genes in these same 5-azacytidine-treated MSB cells. For example, using an embryonic β -globin probe and the same MSB AZA-DNA described above, none of the globin-specific *MspI*-generated fragments is observed with *HpaII* digestion (Fig. 5D). The methylated state of these various globin genes has been maintained in every MSB AZA-DNA sample tested. Thus, the undermethylated state resulting from 5-azacytidine treatment is apparently non-random (see discussion).

An *ev-1* hypersensitive site induced by 5-azacytidine

As mentioned above, regions of DNase I hypersensitivity have been described for several transcriptionally active genes^{7,13–15,37}. Although HMG 14 and 17 are responsible, at least in part, for the preferential sensitivity of each nucleosome within a particular transcription unit³⁸, the basis of the hypersensitivity of the particular point site is unclear (see ref. 39 for extensive discussion). Using β -globin genes, we demonstrated previously that the appearance of these sites coincides with the developmental activation of any one particular β -globin gene. Thus, although no β -globin-hypersensitive sites are observed in precursor haematocytoblasts, such sites are detectable near the embryonic β -globin genes in daughter, first generation erythroblasts¹⁶. The generation of hypersensitive sites during development suggests that these regions are not 'stable' areas in

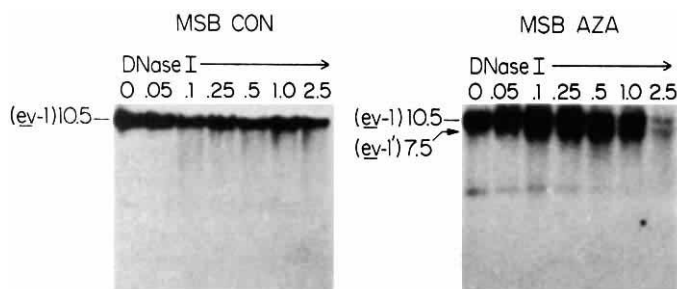


Fig. 6 Appearance of a DNase I-hypersensitive site after treatment of MSB cells with 5-azacytidine. Nuclei from control MSB cells and MSB cells 72 h after exposure to 5-azacytidine were incubated with increasing amounts of DNase I and analysed as described for red blood cell nuclei in Fig. 1. Although no sub-band is detectable in the control cells, a prominent 7.5-kbp sub-band (in addition to the expected 10.5-kbp *ev-1* *Sst*I fragment) is observed in these cells after exposure to the cytosine analogue.

terms of chromosomal conformation, but are the results of processes involved in the developmental activation of particular regions of the chromosome. We wondered, therefore, whether the transcriptional activation of *ev-1* by 5-azacytidine was also correlated with the generation of a hypersensitive site within the chromosomal domain of this retroviral genome. Figure 6 shows the results of low-level DNase I digestions of control and azacytidine-treated MSB cells. Whereas no *ev-1*-related sub-bands are detectable in the *Sst*I-restricted DNA from control cells, a prominent 7.5-kbp sub-band is observed in the DNA isolated from DNase I-digested nuclei of MSB cells, analysed 72 h after exposure to the analogue. In addition, the intensity of the sub-band and the kinetics of its disappearance compared with those of the 10.5-kbp *ev-1* *Sst*I fragment, indicate that the hypersensitive site is present in ~50% of the cells in the azacytidine-treated population. Thus, the activation of *ev-1* by 5-azacytidine is associated with a change in the chromosomal conformation of this gene, paralleling the developmental activation of the chicken globin genes.

Extensive mapping of the azacytidine-induced *ev-1* sub-band (similar to that described in mapping the *ev-3* sub-band) has revealed site-specific double-stranded cuts in both the 3' and 5' LTRs of the endogenous retrovirus (unpublished observations), suggesting that transcriptional activity of two distinct endogenous retroviral loci is associated with a conformational change at a specific sequence located at both ends of these genomes.

Discussion

The experiments described above reveal that the expression of retroviral genomes, like many genes, is correlated with the conformation of these sequences in chromatin. Thus, the unexpressed *ev-1* locus is no more sensitive to DNase I than bulk DNA, whereas the transcriptionally active *ev-3* locus is preferentially sensitive to DNase digestion. In addition, and again as in several other systems, the unexpressed *ev-1* is highly methylated at CCGG residues, whereas the 'active' *ev-3* is relatively undermethylated at these sites. Similarly, although no hypersensitive site is observed on low level DNase digestion of *ev-1*, two hypersensitive sites are detectable (with both DNase I and staphylococcal nuclease) in *ev-3*. These sites are located within the LTR on either side of this retroviral genome. As described by Ju and Skalka²⁷, the LTRs of many retroviruses contain regions of homology with several other genetic elements. Particularly intriguing is a 15/20-bp sequence homology between the retroviral LTRs (CGTCATTCG*ACGATAG-TAG) and a tandemly repeated region in SV40 (CGT**TTCGTACG*TAG*AG), in which this sequence is present in a location recently described as essential for the initiation *in vivo* of early transcription⁴⁰. In addition, this same region of SV40 contains a DNase I-hypersensitive site associated with early transcription (C. Cremisi, unpublished obser-

vation) and is part of a larger region that is preferentially exposed to staphylococcal nuclease and various restriction endonucleases late in infection^{41,42}. The *ev-3* sub-band mapping data presented above tend to localize the *ev-3*-hypersensitive sites to the border of the 5' and 3' sequences within the LTR (see Fig. 3); however, the error in this analysis is such (± 100 bp) that the hypersensitive site could be located in the region of the LTR containing sequence homology with SV40. Several other sequences important in transcriptional control—a TATA box and a cap site—are also located within this 200-bp region of the LTR. Given the location of such sites in both LTRs, we believe that a particular DNA sequence or sequences dictates the chromosomal conformation revealed by the DNase I-hypersensitive sites. Clearly, other factors (such as the pattern of DNA methylation) are also important because in normal chicken cells the LTRs present in *ev-1* are not hypersensitive even though they contain sequences with the potential for generating hypersensitive regions, as evidenced by the appearance of such sites after treatment with azacytidine. Recently, Blair *et al.*⁴³ have shown that ligation of LTRs to either the 5' or the 3' side of a virus-transforming gene increased the frequency of morphologically transformed colonies after DNA transfection. In the context of the present experiments, these results suggest that LTRs increase transcription not only by providing a high-efficiency 'promoter'⁴⁴, but also by 'opening-up' large tracts or domains of chromatin structure, possibly by phasing adjacent nucleosomes or segregating genes into a specific nuclear compartment required for transcription. If this is so, and if the LTR signal for this proposed change in chromatin structure is bidirectional (as its many inverted repeats suggest), it would not be surprising if these sequences have an effect when placed either 5' or 3' to a given gene. Although the biochemical basis for the hypersensitivity is unknown, these sites may represent regions of specific interactions with DNA sequence recognizing proteins. DNA 'footprinting' analysis of several DNA binding proteins has shown that sites within the binding domain of the protein can be 10–100-fold more sensitive to DNase I cutting when the protein is bound⁴⁵.

We have also demonstrated that 5-azacytidine-induced undermethylation of the unexpressed *ev-1* results in the transcriptional activation of this retroviral genome. In contrast to the induction of murine proviruses by such agents as BUdR and IUdR, induction of *ev-1* in chickens by halogenated pyrimidines or other agents has not been demonstrated. However, several experiments^{46,47}, and our preliminary results, indicate that *ev-1* cannot be efficiently induced by such agents. Although our azacytidine results strongly suggest that *ev-1* activation is effected through a change in the methylated state of DNA, this has not been proved (for example, by DNA-mediated transfer). However, it is supported by the evidence presented above (change in cutting at *Hpa*II sites; requirement for DNA synthesis; and generation of hypersensitive sites) and by the findings that ethionine and 3-deazaadenosine, two very different types of analogues which affect DNA methylation, also induce gene activity^{48,49}. Nevertheless, at present there is no direct experiment to exclude the possibility that azacytidine leads to the activation of some other cellular locus which, in turn, activates the *ev-1* locus. In this regard, results of transfection experiments in murine cells indicate that halogenated pyrimidines can affect viral gene transcription by acting at a site other than the viral genome⁵⁰. Our inability to observe 5-azacytidine-induced changes in the methylated state of several globin genes in the same cells in which regions of *ev-1* are clearly undermethylated could be interpreted to support such a *trans* effect, assuming that the 'target size' for the putative regulating locus of *ev-1* is much larger than that for the entire globin domain. Alternatively, it seems more reasonable that initially all genes are equivalently undermethylated, but that some type of selective correction mechanism leads to the re-methylation of the globin genes. Finally, the observed selective undermethylation of *ev-1* in the AZA-MSB cells could be due to a non-random incorporation of the analogue. All these possibilities are being investigated.

The role of DNA methylation and gene function has been studied by several laboratories and recently reviewed by Razin and Riggs⁵¹. Our own work focuses on a particular gene and also suggests that undermethylation is important in generating a chromosomal structure permissive for transcription, for the transcriptional activation of *ev-1* accompanies the appearance of a hypersensitive site, similar in location to that point site associated with the transcriptionally active *ev-3*. Our experiments seem to suggest that undermethylation of all or a specific subset of CpG dinucleotide sites within the chromosomal domain of a given gene precedes the acquisition in chromatin by that gene of a conformational change permissive for transcription. It seems useful to compare our results on the activation of *ev-1* with those of Jones and Taylor, who monitored cytodifferentiation, the presumed activation of entire programmes for gene activity. Using roughly comparable degrees of exposure to azacytidine, we estimate that at least 50% of the *ev-1* loci are activated (see above), whereas Jones and Taylor observed about 1% of the cells in their cultures differentiating, for example, into muscle cells. We presume that the differences in frequency reflect the relative sizes of the respective targets needed to be substituted for the two assays. This could

represent a requirement for greater substitution in a single target for myogenesis or, more likely, a requirement to activate more genes before muscle cytodifferentiation can be scored.

Although the examples of chicken α - and β -globin genes^{5,11}, and the active retroviral genomes described above, show a striking correlation between gene expression and hypomethylation of almost all the *HpaII* sites in a transcription unit, most studies in other systems do not support such a simple correlation (for example, ref. 10). Thus, we feel that undermethylation of specific regions of a locus^{6,9,10} may be the signal important in generating an active chromatin structure. This hypothesis predicts that exposure of MSB cells to very low levels of 5-azacytidine should result in the transcriptional activation of *ev-1* in a subpopulation of the cells undermethylated only at one site or a few sites, possibly within the LTR. Analysis of subclones of such cells would then be expected also to reveal a specific hypersensitive site.

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LETTERS TO NATURE

Peculiar optical spectrum of the Red Rectangle

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The Red Rectangle¹ is a nebula centred on the star HD44179. Within an amorphous blue nebula is embedded a hollow biconical red nebula with apex at the star. The generators of the hollow bicone show in projection as four spikes radiating from HD44179. The bicone has a hitherto totally unidentified spectrum. In high resolution spectra of the nebula we have identified a narrow-line component (Na D, Ca H and K, H α) from a low excitation plasma and we have resolved broader features presumably from molecular bands. Here we give constraints on the molecules responsible, and propose as a candidate carbyne (linear chains of carbon with alternate single and triple bonds). The Red Rectangle may thus be a factory for the production of the raw material from which some interstellar molecules are made.

Low resolution (50 Å) spectra² of the Red Rectangle show a blue continuum spectrum identical to that of the central star, plus a broad red emission feature which extends over 2,100 Å and is centred near 6,400 Å. From polarization measurements^{3,4} it is clear that, while the blue spectrum is scattered star light from HD44179, the red spectrum is emission originating on the bicone. Polarization studies indicate⁴ that the bicone is formed of a concentration of dust grains as well as the unknown ingredient X_{RR} responsible for the red emission feature. Moderate resolution spectra (10 Å) break³ the feature into considerable structure, including resolved components grouped like molecular band-heads, presumably from a molecule X_{RR} in association with the dust grains which give the polarization behaviour. IR spectra^{1,5} of HD44179 itself show emission bands also usually seen in association with dust, but not the 9.7- μ m feature which is a signature of silicates.

We have taken high resolution (1 Å) spectra of the Red Rectangle on the 3.9-m Anglo-Australian Telescope in several positions in the nebula, and spectra of the central star, in the wavelength range λ 3,400–6,600. Figure 1 illustrates the spatially summed spectrum of the nebula in the wavelength range λ 5,700–6,660, which includes most of the red emission feature. Figure 2 illustrates the blue spectrum 3,400–4,400 Å which shows prominently the Balmer absorption lines in the reflection spectrum.

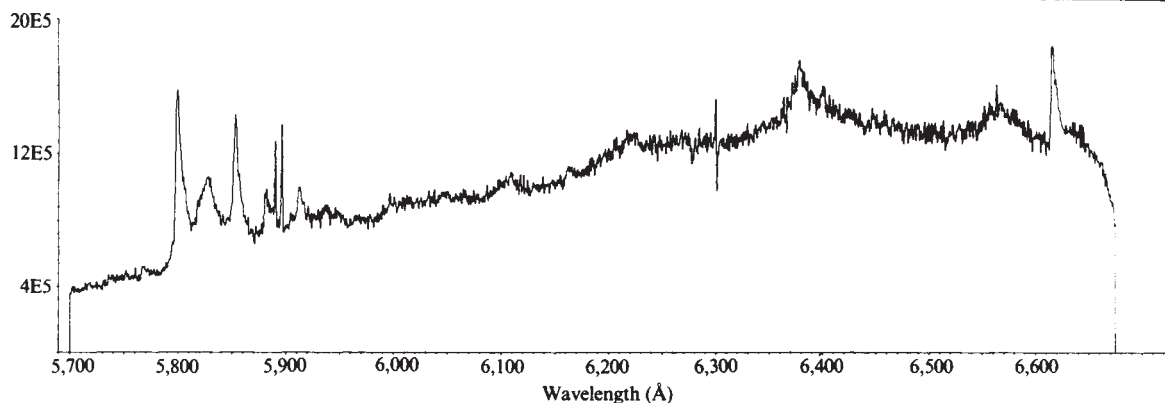


Fig. 1 Spectrum of section 1.2 arc s wide across the nebula, centred 7 arc s north of HD44179 at position angle 120° , as observed for 10,000 s with 0.5 Å spectral elements. Ordinate represents photons detectable per element in a perfectly efficient 3.9-m telescope. The D lines and $H\alpha$ are superimposed on a banded spectrum. (The feature at 6,300 Å is from night-sky emission.)

In these spectra of the nebulosity there are four distinct types of emission features superimposed on the scattered stellar continuum:

(1) The nebula contains narrow emission lines at the wavelengths Na D (strong), $H\alpha$ (weak), and Ca II H and K (strong). The central star has strong narrow emission lines of Na D and strong $H\alpha$ emission, but no detectable H and K emission; on the contrary, there is weak K-line absorption, perhaps interstellar. Thus these nebular emission lines are not scattered starlight but are intrinsic emission from the nebula. Indeed, the ratio of the Na D lines varies across the nebula, being $\sim 2:1$ (optically thin) just outside and within the bicone but $\sim 1:1$ (optically thick) on the spikes, thus confirming the hollow structure of the bicone. There is no detectable broadening or splitting or velocity shift of the sodium lines across the nebula, and we estimate that projected bulk motions in this plasma are less than 15 km s^{-1} , and turbulent motion has a FWHM less than 35 km s^{-1} . The narrow emission lines are from a very low excitation plasma. The ionization potentials of Na I and Ca II are 5.1 and 11.9 eV; the weak $H\alpha$ indicates that ionization energies just up to 13.6 eV are available. The mean surface brightness of $H\alpha$ over the part of the nebula which we measured is $6.9 \times 10^5 \text{ photons s}^{-1} \text{ sr}^{-1} \text{ cm}^{-2}$ which is consistent with ionization by the AO-B8 II-III star HD44179 (Strömgren sphere of 1–2 pc diameter) of $3\text{--}8 \times 10^{-6}$ solar masses of hydrogen (distance¹ of 330 pc, angular size = 0.5 arc min FWHM) with r.m.s. density in the range $40\text{--}100 \text{ cm}^{-3}$. The $H\alpha$ detection is consistent with upper limits to radio detection¹ at 8.4 and 15 GHz of $\sim 0.05 \text{ Jy}$. The low excitation spectrum of the nebula indicates that its temperature is lower than the canonical 10^4 K of a typical H II region. Other low temperature H II regions⁶, which may be cooled by abundance anomalies⁷ (metal rich),

have temperatures in the region of 4,000 K, and judging by its low ionization, the Red Rectangle may be cooler still.

The other three types of feature in the spectrum of the Red Rectangle are difficult to identify.

(2) There is the very broad feature, which ranges³ from $\lambda 5,400$ to $7,500$.

(3) There are *R*-type band-like features characterized by a sharp rise from the shorter wavelength and a gradual degradation to the red⁸; these occur at $\lambda 5,799, 5,855, 5,880$ and $6,615$, the first two and the last one being most prominent. All these features have very similar structure and their relative intensities remain reasonably constant with position in the nebula. However, the degree of degradation to the red seems to decrease on the spikes at the edge of the biconical nebula. No velocity structure, as measured by the peak of the *R*-type features, can be seen over the nebula, to an accuracy of 50 km s^{-1} .

(4) The emission from the Red Rectangle also contains *M*-type diffuse features that are broad ($10\text{--}20 \text{ Å}$ FWHM), peaked in the middle and centred at $\lambda 5,828, 5,913, 5,938, 6,109, 6,208, 6,378, 6,420, 6,563$. There may be weak features in the blue spectrum but because of low signal-to-noise and confusion with the Balmer series we cannot identify them well; we note the possible feature at $4,044\text{--}4,053$.

The ingredient X_{RR} which gives rise to all these features may be simple molecules trapped on the grains abundant within the nebula, in which case the mode of attachment may modify the emission wavelengths and a comparison with laboratory spectra of 'free' molecules would not yield an identification. Alternatively the emission may be from the grains themselves undergoing a form of fluorescence as a result of some illumination from the central star, although no outstanding stellar spec-

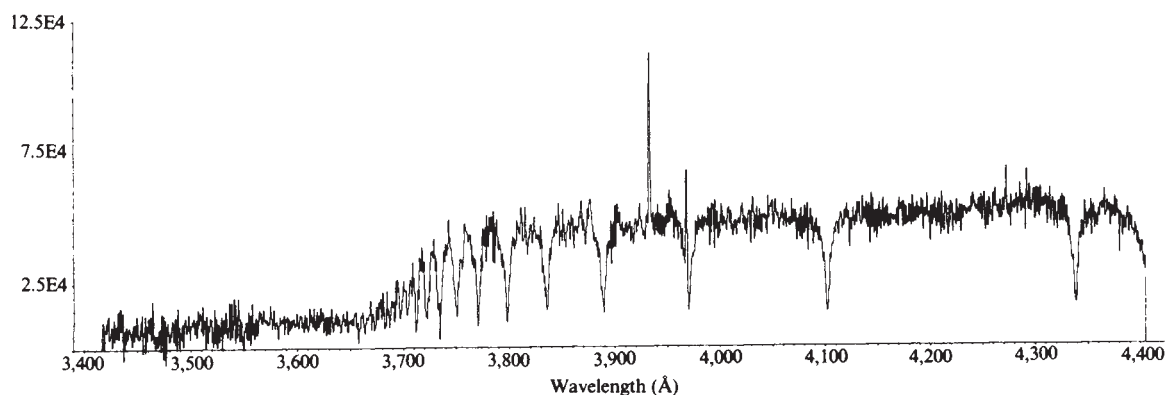


Fig. 2 Spectrum of the blue region of the nebular spectrum showing H and K emission superimposed on the Balmer line continuum of scattered star light. Ordinate as Fig. 1, for 2,000 s integration.

tral component for this illumination can be identified. It is more likely³ that ingredient X_{RR} consists of free molecules in the nebular medium.

If the features are molecular emission lines³ then the very broad emission feature (2) could correspond to molecular dissociation, and features (3) and (4) could be transitions between vibrational states of different electronic states of the molecule. The diffuse or degraded structure could correspond to rotational broadening, and changes in the degradation from place to place correspond to a difference in excitation temperature. The question arises as to which molecule X_{RR} might be.

There are some coincidences³ between the spectrum of the Red Rectangle and that of H_2O^+ (as found in comets⁹) and of C_3 but in each case there are additional spectral lines of these molecules that are absent from the spectra. The C_3 series is the better fit because in the laboratory it produces phosphorescent lines at 5,856, 5,874, 5,911, 5,969 and 6,308 Å in a neon trapping gas, with a 50-Å shift to the red in argon¹⁰. A 57-Å shift to the blue (in an arbitrary attempt to simulate the environment in the Red Rectangle) produces 5,799, 5,817, 5,854, 5,912 and 6,251 Å which may be identified with the X_{RR} transitions at 5,799, 5,828?, 5,855, 5,912 and 6,208?? Å but does not identify the third strongest band at 6,615. It is tempting to identify the weak blue feature at 4,050 Å with the strongest of the Swings' band of C_3 , and this would indicate that there may be limited regions in the Red Rectangle with temperature $\leq 3,000$ K.

There is, however, a general similarity between the spectrum of the Red Rectangle and those of some open-shell organic cations studied by Maier¹¹. They have a broad (~2,000 Å) dissociation spectrum in the red (5,000–8,000 Å region for the examples given) and red degraded or middle-peaked bands which are strongest on the blue end of the broad feature. Common building blocks of many of these linear organic molecules are the $C\equiv C$ and $C-C$ bonds. Maier¹¹ gives the vibrational frequencies of the stretching fundamentals of the $C\equiv C$ and $C-C$ bonds as 2,119 and 629 cm^{-1} for the ground molecular state of dicyanoacetylene. These frequencies are not significantly different ($\pm 20\%$) for the same bonds in other linear cations quoted by him or for the neutral molecules which we anticipate will be present in the Red Rectangle. It so happens that in the Red Rectangle spectrum the stronger bands at λ 5,799 and λ 6,615 have an energy difference of 2,127 cm^{-1} and the bands at λ 5,880 and λ 6,109 have a difference of 638 cm^{-1} . As both these energy differences in X_{RR} correspond well with those of the $C\equiv C$ and $C-C$ bond stretching frequencies, it is tempting to identify X_{RR} with an unknown linear organic molecule that is constituted in part by $C\equiv C$ and $C-C$ configurations. Linear carbon chain molecules have low transverse vibrational frequencies and these may also contribute in addition to rotational effects to the detailed structure of the observed band-like features.

Many of the organic molecules found in the interstellar medium contain some such configurations, for example cyanoacetylene^{12,13} ($H-C\equiv C-C\equiv N$). Carbyne, which has been proposed¹⁴ as a constituent of interstellar material, is a linear structure of carbon with strings of $C-C\equiv C$ chains. Webster¹⁴ attributes some of the unidentified IR bands between 3.27 and 11.3 μm to carbyne; they are carried by something that is carbon-rich^{15,16} and are present⁵ in HD44179. Carbyne is stable¹⁷ between 2,600 K and 3,800 K, consistent with the temperature of the narrow-line plasma in which the bicone is bathed. Carbyne might be produced¹⁷ above ~3,000 K from C_3 molecules ($C=C=C$) by transfer of one bond and/or from graphite grains by fracturing the hexagonal planes of which graphite is composed. We have pointed already to the marginal evidence for the 4,050 Swings' C_3 band in the Red Rectangle and to the absence of the 9.7- μm feature in the grains in HD44179 so that in the absence of silicate grains^{15,16} we therefore suppose they may be graphite in an oxygen-deficient environment. It is thus conceivable that carbon phases associated with carbyne are present in the Red Rectangle, and it is a likely candidate for X_{RR} although in the absence of a reference

spectrum the case is circumstantial. We cannot exclude other molecules as we have no explanations for several features in the spectra.

The formation of a linear molecule such as carbyne in the nebular environment around stars like the Red Rectangle may be an important initial step in the production of the many organic molecules found in the interstellar medium.

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Inverse comptonization and the nature of the March 1979 γ -ray burst event

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The central issue concerning the nature of the 5 March 1979 γ -ray burst is that of whether its source is the supernova remnant N49 in the Large Magellanic Cloud (LMC), whose extragalactic distance implies unusually high (super-Eddington) luminosity. Although the optically thin synchrotron mechanism proposed by Ramaty *et al.*¹ has met many of the earlier theoretical objections, other evidence is needed to support or disprove such an extragalactic origin. I point out here that the observed² burst spectrum seems to be most naturally and consistently interpreted as that of a synchrotron spectrum modified by inverse Compton scattering from $\sim MeV e^+e^-$ pairs. (Inverse Comptonization describes the gain of energy by photons as a result of scattering with electrons of much higher energy.) This model then allows us to derive, from first principles, the intrinsic synchrotron luminosity of the burst source, which agrees basically with that expected from N49 (distance ~ 55 kpc).

The model spectrum of Ramaty *et al.*¹ suggests that the spectral density above ~ 400 keV is greater than that from a simple redshifted pair annihilation line (Fig. 1). On the other hand, a power-law tail with a high energy cutoff (at 1.5–2 MeV in the present case, Fig. 1) is produced when a copious supply of soft photons (the synchrotron X rays below ~ 300 keV in the present case) is made more energetic by Compton scattering from hot electrons, provided that the Comptonization is unsaturated (the average fractional energy gain of the emerging photons $\equiv \gamma \leq 1$)^{3–5}. This has become the normal interpretation of the X-ray spectra of Cyg X-1 and of active galaxies. It is evident that the same relativistic pairs that are emitting the synchrotron X rays in the model of Ramaty *et al.*¹ would automatically also Compton upscatter some of them, despite the low scattering depth. Hence, the dominant contribution to the γ intensity above the break at ~ 300 keV (Fig. 1) is taken here to be comptonized photons, with only a small admixture of redshifted 511-keV photons producing the bump at 430 keV

(ref. 2). Uncertainty in the annihilation photon contribution does not matter in our estimates as long as it is smaller than the continuum. But, to be specific, the Compton continuum is taken as that lying below the dashed line in Fig. 1, with the excess due to the line contribution. The dashed line is the best fit to the data based on the general form of the unsaturated inverse Compton spectrum^{3,6} (see below).

In the limit when comptonization is highly unsaturated ($y \equiv$ Kompaneet parameter $\ll 1$)³⁻⁵, which is evidently the case here, the ratio of the hard comptonized energy flux F_C to the uncomptonized soft flux F_S is simply $F_C/F_S \approx y$ (refs 3, 5). Breaking the spectrum at ~ 300 keV (Fig. 1: S denotes synchrotron component; C, Compton component) we find

$$y \equiv \frac{F_C}{F_S} \approx 0.077 \quad (1)$$

Also, in the optically thin limit ($\tau_{es} \ll 1$), the ratio of comptonized photons N_C to uncomptonized photons N_S is the mean scattering probability:

$$\tau_{es} \equiv \frac{N_C}{N_S} \approx 6.5 \times 10^{-3} \quad (2)$$

(from Fig. 1). In the relativistic regime^{7,8},

$$y = \frac{4}{3} \tau_{es} \Gamma^2$$

where $\Gamma \equiv \bar{E}/m_e c^2$ is the average relativistic factor of the pairs. Hence,

$$\Gamma^2 \approx 8.9 \quad \text{or} \quad \Gamma \approx 3 \quad (3)$$

This is consistent with the apparent steepening of the tail above ~ 1 MeV. Pozdnyakov *et al.*⁶ have derived the spectral index of the power law tail for relativistic comptonization. The effective spectral index in the present case is⁶

$$\alpha = -\frac{\ln \tau_{es}}{\ln (4\Gamma^2/3)} \approx 2.04 \quad (4)$$

using equations (2) and (3). Hence, the predicted photon number spectrum is in approximate agreement with the dashed curve (ref. 6, Fig. 4).

From equation (2), we find the pair column density

$$n_e h \equiv (n_+ + n_-)h = \frac{\tau_{es}}{\sigma_{KN}} = 3.42 \times 10^{22} \text{ cm}^{-2} \quad (5)$$

where the Klein-Nishina cross-section $\sigma_{KN} \approx 1.9 \times 10^{25} \text{ cm}^2$ at $\Gamma \approx 3$. The soft component is essentially an exponential of decay constant ~ 20 to 30 keV. Interpreting this as the (redshifted) critical synchrotron frequency^{7,8}, we have

$$\begin{aligned} h\nu_c &\equiv 1.78 \times 10^{-11} B_{\text{Gauss}} \Gamma^2 \text{ keV} \\ &\approx 30 \text{ keV} \end{aligned} \quad (6)$$

Hence,

$$B \approx 1.9 \times 10^{11} \text{ G} \quad (7)$$

consistent with the field expected at a typical neutron star surface. From equation (5) and (7), we derive the intrinsic synchrotron luminosity

$$\begin{aligned} L_{\text{syn}} &= 2.3 \times 10^{-2} B^2 \Gamma^2 n_e h f_* \\ &= f_* 2.5 \times 10^{44} \text{ erg s}^{-1} \end{aligned} \quad (8)$$

where f_* is the fraction of the surface area of a 15-km neutron star that is emitting. This (optically thin) synchrotron luminosity, which is derived without any reference to the assumed distance to the source, is so close to the quoted burst luminosity of $\sim 3 \times 10^{44} \text{ erg s}^{-1}$ at the distance of N49 that it serves as a consistency check on the LMC identification (f_* is expected to be of the order unity from the width of the subsequent 8-s period pulses²). The mean synchrotron self-absorption depth is $\bar{\tau}_{\text{syn}} \sim 10^{-4}$, completely justifying the optically thin assumption (used in equation (8)). In fact, the frequency at which the self-absorption depth approaches 1 is ~ 20 keV, consistent with the obser-

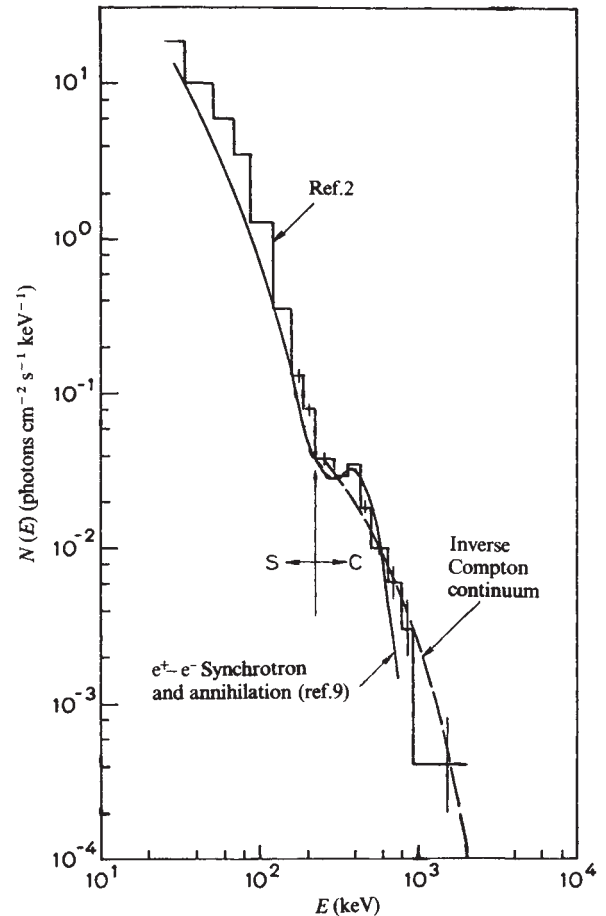


Fig. 1 Comparison of the models of the 5 March 1979 γ -ray transient, according to Mazets² and Ramaty *et al.*⁹ with the present inverse Compton continuum.

ved low-energy turnover. The synchrotron cooling time of the relativistic pairs is

$$t_{\text{syn cool}} \approx \frac{6 \times 10^8 \text{ s}}{B^2 \Gamma} \approx 2 \times 10^{-15} \text{ s} \quad (9)$$

much shorter than bremsstrahlung cooling time or pair annihilation time. Hence, the pairs cool before annihilation, as expected¹. However, their probability of Compton-upscattering soft photons before cooling down is still high because of the large amount of soft photons present:

$$t_{es} = \frac{1}{n_+ \sigma_{KN} c} \sim 3 \times 10^{-15} \text{ s}$$

Equation (9) also indicates that the long duration of the burst (~ 120 ms) must be supported by a continuous supply of relativistic pairs which may be produced from slow dissipation of large energy reserves (for example, neutron star vibrations of ref. 9).

If we identify all the excess above the dashed line continuum in Fig. 1 as coming from pair annihilation, we find (now assuming a true distance of 55 kpc) for the pair annihilation luminosity

$$L \approx 1.8 \times 10^{41} \text{ erg s}^{-1} \quad (10)$$

or

$$n_+ n_- h f_* \approx 1.4 \times 10^{48} \quad (11)$$

Assuming $n_+ \approx n_-$, we have, combining equations (11) and (5),

$$n_+ \approx 10^{26} f_*^{-1} \text{ cm}^{-3}$$

and

$$h \approx 2 \times 10^{-4} f_* \text{ cm} \quad (12)$$

These numbers are generally similar to those of Ramaty *et al.*¹ One clue as to why h is so small may be the extremely short

cooling time of the relativistic pairs which limits the distance over which a newly created pair can traverse before cooling down ($ct_{\text{syn cool}} \approx 6 \times 10^{-5}$ cm). Of course, we still need to explain why all pairs must be created in such a thin layer. As with all models dealing with an LMC origin, this model still faces problems related to the huge energy source, fast coupling, and the slow decay of the subsequent pulsations. Of course, interesting ideas have already been proposed (see ref. 9), but detailed analysis remains to be done.

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Microanalysis by Raman spectroscopy of carbon in the Tieschitz chondrite

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The Tieschitz H3 chondrite contains 0.25% bulk carbon¹ but concentrations of as much as 2–6% (ref. 2) occur in black, 10–20 μm -wide rims around chondrules that are thought to have accreted before the agglomeration of the stone^{2–5}. These rims are extremely fine-grained and compact, the main constituent being an iron-rich olivine (Fa 60–65) as shown by a Debye–Sherrer X-ray diffractogram, in good agreement with the FeO/FeO+MgO matrix value^{2,6}. To further our understanding of the rim composition and of the thermal history of the stone, we used the MOLE Raman laser microprobe⁷ to characterize the carbon constituent. We report here that it is a highly disordered graphite with a progressive ordering as shown by samples heated to temperatures between 300 and 600 °C. Tieschitz could not have been heated to more than 300–350 °C.

Polished sections were prepared from Tieschitz samples previously heated in a nitrogen flux to 300 and 350 °C for 2 h, and to 400 and 600 °C for 1 h. These four samples were then compared with a section of Tieschitz that had not been heated. Areas of interest were precisely located under the microscope and recorded on photomicrographs. The specimens were then transferred to the MOLE microprobe (Jobin–Yvon). The power of the 5,145-Å line of a Spectraphysics 160 argon ion laser is 10 mW at the sample surface, thus the laser beam was slightly defocused to avoid degradation of the sample during analysis and the resulting excited area was $\sim 10 \mu\text{m}^2$. The nominal magnification used was $\times 1,000$ and width of the spectral slit was 6 cm^{-1} . As the width of the observed bands was large, the precision of the reported frequencies is $\pm 5 \text{ cm}^{-1}$. The reported relative intensities were not corrected for the spectrometer yield, thus the $1,605 \text{ cm}^{-1}$ line is underestimated by $\sim 3\%$ compared with the $1,355 \text{ cm}^{-1}$ line.

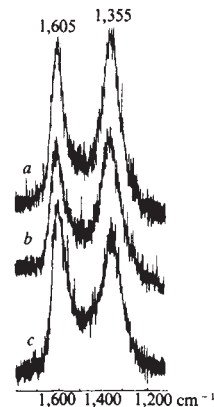


Fig. 1 Raman spectra between 1,100 and 1,800 cm^{-1} of chondrule rims in the Tieschitz H3 chondrite; a, unheated sample; b, sample heated to 300 °C; c sample heated to 600 °C.

In the 2,500–1,000 cm^{-1} region, the monocrystalline graphite Raman spectrum exhibits a single band at $1,575 \text{ cm}^{-1}$, whereas polycrystalline graphite and more or less disordered carbon materials show two main bands at $1,605$ and $1,355 \text{ cm}^{-1}$, the relative intensities of which depend on the degree of 'graphitization'^{8–10}. The $1,605 \text{ cm}^{-1}$ band, deriving from crystallized graphite, is related to vibrations in the layers. The $1,355 \text{ cm}^{-1}$ band may be assigned to distortion of the hexagonal lattice. The relative intensity ratio of the two bands $\rho = I(1,605)/I(1,355)$ can thus be used as a measure of the imperfection of the graphite layer planes. This was done for carbons heat-treated temperatures between 1,000 and 2,500 °C^{9,10}. A relation between ρ and the size of the carbon particles was also found⁸, although it remains to be established whether this is a causal relationship.

About 10 carbon-rich rim areas were studied for each of the Tieschitz sections between 900 and 2,300 cm^{-1} . No detectable degradation of the sample was observed during recording of spectra. To monitor possible effects of degradation, each spectrum was registered in a symmetric mode, sweeping the relevant frequencies both ways. In all cases studied, only the two bands of more or less graphitized carbon were found (Fig. 1). Carbynes, the occurrence of which is questioned in carbonaceous chondrites^{11–13} were not detected here.

The relative intensity ratio ρ , determined for each specimen over 10 spectra, shows a deviation of $< 4\%$ with respect to the mean. This should be ascribed to experimental errors (because of the rather low signal-to-noise ratio) rather than to the existence of real variations. For unheated Tieschitz $\rho = 0.92 \pm 0.03$. This ratio increases with increasing temperature for the heat-treated specimens, and thus constitutes a convenient index for the degree of crystallinity of the sample. For $t = 300, 350, 400$ and 600°C , $\rho = 0.93, 0.97, 1.05$ and 1.23 , respectively. In the relatively narrow temperature range investigated, ρ increased linearly with t (Fig. 2). The ρ for unheated Tieschitz is almost indistinguishable from that of the sample heated to 300 °C. We can therefore conclude that Tieschitz, after accretion, was not heated above 300–350 °C. Moreover, the ρ value of 0.92 for the untreated specimen allows us to give an approximate 40 Å limit to the size of the graphite layers occurring in Tieschitz chondrule rims; this corresponds either to crystallite size, or to the size of undisturbed domains. These small particles are dispersed in the entire rim, as the characteristic Raman spectra are identical at any point on the rims.

Independent confirmation of the 300–350 °C heating is given by the nickel–iron alloys that occur in Tieschitz: (1) according to Bevan and Axon¹⁴, interface Ni compositions of contiguous kamacite and taenite grains indicate equilibration to $\sim 350^\circ\text{C}$; (2) tetrataenite—the ordered 50% Ni–Fe alloy¹⁵—occurs either as rare isolated grains (their anisotropy is then observable), or in zoned taenite as a clear rim and in association with nickel-poor

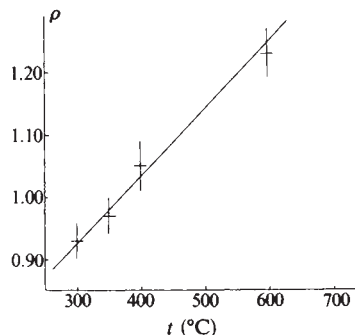


Fig. 2 Intensity ratio of Raman bands at 1,605 and 1,355 cm^{-1} , $\rho = I_{1,605}/I_{1,355}$ obtained on chondrule rims, plotted against temperature.

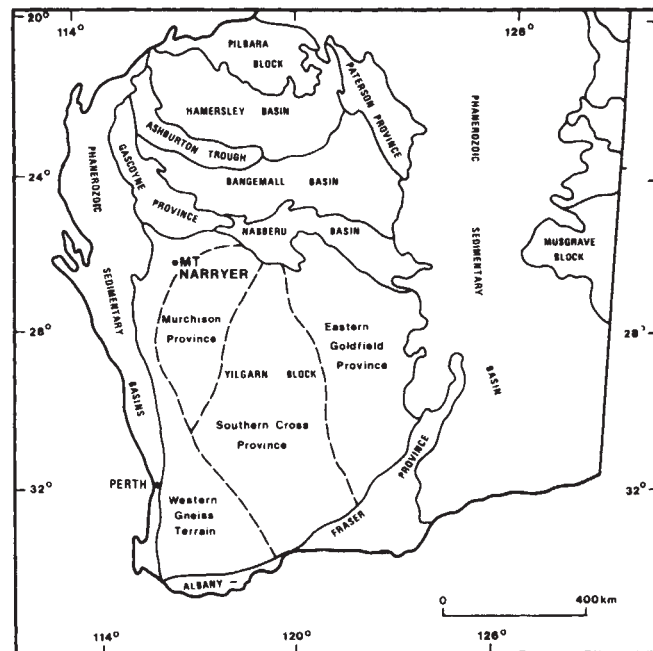


Fig. 1 Distribution of Precambrian tectonic units in relation to Mount Narryer, Western Australia.

alloys, giving a cloudy appearance to etched zones. The ordering of this alloy may occur below 320°C and is only observed in slowly-cooled meteorites.

Further microanalyses by Raman spectroscopy of carbon in meteorites will hopefully help us to understand the complex and controversial thermal history of these extraterrestrial rocks.

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Early Archaean gneisses from the Yilgarn Block, Western Australia

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The Yilgarn Block is an ancient crustal block which extends over ~650,000 km^2 in the south-west of Western Australia and consists of high-grade gneiss and granite-greenstone terrains¹. Being one of the largest segments of Archaean crust in the world, it is important to studies of early crustal evolution. We report here a geochronological study of banded gneisses near Mount Narryer, in the north-west of the Yilgarn Block, which has given a Rb–Sr whole-rock isochron age of $3,348 \pm 43$ Myr with an initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7037 ± 0.0005 . The data indicate that the rocks had a prior crustal residence time of ~200 Myr. The validity of this early Archaean age is supported by model Sm–Nd ages of 3,510 Myr and 3,630 Myr for two of the samples. Taken together, these ages represent the oldest evolutionary sequence so far identified in the Yilgarn Block, and are comparable with old ages from other Archaean cratons elsewhere in the world.

Contrasting evolutionary models have been applied to the regional patterns of the Yilgarn Block. One model interprets the gneiss terrains as the metamorphosed and migmatized equivalents of the adjacent greenstones, infolded to deep crustal levels²; the other interprets the gneiss terrains as basement complexes on which greenstones were deposited^{3,4}. However, this debate⁵ is by no means settled, either in the Yilgarn Block, or in other well studied Archaean cratons in the world.

It has been established¹ that the gneisses of the Yilgarn Block occur mainly in an arc >1,000 km long along the western margin of the block, termed the Western Gneiss Terrain (Fig. 1). It was concluded on geological and equivocal geochronological evidence that the gneisses were old basement, and that the greenstone-granite terrains evolved in response to a widespread but relatively short-lived tectonothermal event ~2,700 Myr ago.

The importance of the Yilgarn Block as a reference for early crustal evolution further lies in the nature of the Western Gneiss Terrain. In addition to the extensive areas of banded orthogneiss, there are occurrences of paragneiss in which quartzite, BIF, metacarbonates, metapelite and metaconglomerate occur. The paragneiss is intruded by mafic and ultramafic rocks, now also highly metamorphosed, but with no identifiable volcanics. The paragneiss is clearly 'non-greenstone' in character, and points to sedimentary regimes in the earliest geological record, more uniformitarian than the volcanic regimes of the later greenstones, widely considered to be unique to the Archaean.

The extensive geochronological data for the Yilgarn Block have recently been reviewed⁶. The isotopic dates for most Yilgarn granitoids vary little from the range 2,600–2,700 Myr, and all Rb–Sr ages to date indicate ages for the greenstones in this range, although it has not been unequivocally established that these are volcanic ages as opposed to alteration ages. Early Rb–Sr studies⁷ had indicated that the gneisses near the western margin of the Yilgarn Block may be older than 3,000 Myr. This is confirmed by the present work, and also by zircon U–Pb dates of 3,340 and 3,250 Myr from quartzite and orthogneiss in the Toodyay area, ~90 km east of Perth⁸.

The Rb–Sr data for the samples collected from the Mount Narryer area (26°27' S, 116°24'30" E), in the northern part of the Western Gneiss Terrain, is listed in Table 1 and plotted on an isochron diagram in Fig. 2. A least-squares regression of the data points, using the method of McIntyre *et al.*⁹, yields a slope and intercept corresponding to an age and initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of $3,348 \pm 12$ Myr and 0.70383 ± 0.00008 (2σ errors), with a

MSWD = 10.8. The relatively large value for the MSWD indicates that the data do not fit within experimental error, and that some isotopic remobilization may have occurred. Therefore a more realistic evaluation of the age and initial ratio of the banded gneiss is that provided by model 4 (ref. 9), which gives $3,348 \pm 43$ Myr and 0.7037 ± 0.0005 (2σ errors).

The samples were collected from a site ~13 km due west of Manfred Homestead and 9 km north-northeast of Mount Narryer 'trig' point. The Mount Narryer rocks are a high-grade amphibolite-granulite transition facies metasedimentary sequence. All samples are banded quartz-feldspar-biotite gneiss of granodiorite to adamellite composition. Microcline is present in all samples and plagioclase exists as oligoclase. Samples rich in quartz and microcline contain metamict allanite. All samples have been reformed and recrystallized beyond the primary gneissose structure and in part are protomylonitic. Plagioclase is lightly saussuritized and biotite has been recrystallized and in part chloritized in the course of reformation of the gneiss. Sample 60739 is largely epidote and has been excluded from the isochron.

Strontium evolution analysis of the data from Mount Narryer suggests a mantle evolution age of 3,550 Myr assuming single-stage evolution. This value was calculated from the determined age (3,348 Myr), the initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio (0.7037) and the arithmetic mean of measured $^{87}\text{Rb}/^{86}\text{Sr}$ ratios (1.32). Mantle Sr evolution was assumed to be linear from 0.6990 at 4,600 Myr to 0.7037 at present¹⁰. The projected path of strontium isotope evolution in the sample intersects the modelled mantle development curve at 3,550 Myr at a $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7000. An independent determination of 0.700 for the initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of a 3,350 Myr gneiss from the South Indian Craton¹¹ suggests that the mantle model which was used is realistic. On these assumptions, prior crustal residence of ~200 Myr is suggested.

As a test of the hypothesis that the gneisses at Mount Narryer had a substantial crustal residence time before the effects of dynamic metamorphism reset the Rb-Sr isochron at 3,348 Myr, Sm-Nd model ages were determined for two of the samples in the suite, as this geochronological technique has some advantages over the Rb-Sr method in determining ages of differentiation of crustal rocks from the mantle. The analytical data are given in Table 2.

The model $T_{\text{Chur}}^{\text{Nd}}$ ages of 3,510 Myr and 3,630 Myr are consistent with a crustal residence time of ~200 Myr for the gneisses that yield the Rb-Sr whole-rock isochron age of 3,348 Myr. The earlier ages are interpreted as the time of extraction of quartzo-feldspathic material, probably as granitic melts from the mantle; and the later age is taken to represent the age of the gneiss that was derived by metamorphic reworking of the primeval quartzo-feldspathic material.

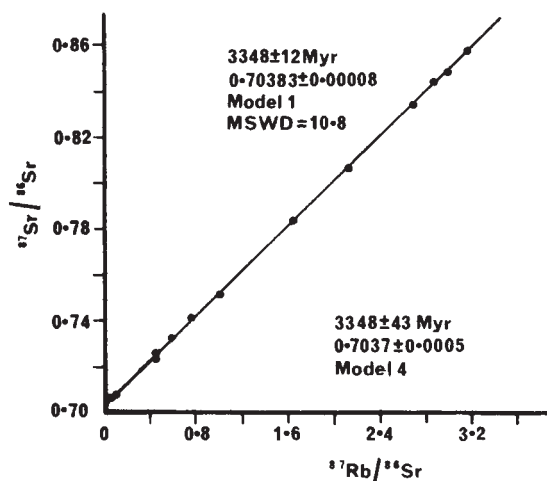


Fig. 2 Mount Narryer, Rb-Sr whole-rock isochron. Errors are quoted at 2σ level⁹.

Table 1 Rubidium strontium analytical data, Mount Narryer

Sample	Rb	Sr	Rb/Sr	$^{87}\text{Rb}/^{86}\text{Sr}$	$^{87}\text{Sr}/^{86}\text{Sr}$
60739*	33	670	0.0045	0.013 ± 0.001	0.71948 ± 9
60734	6.7	310	0.022	0.064 ± 0.001	0.70703 ± 12
60736	17	680	0.024	0.069 ± 0.001	0.70720 ± 7
60737	60	387	0.150	0.433 ± 0.005	0.72439 ± 12
69634	71	485	0.154	0.445 ± 0.005	0.72503 ± 11
69626	61	313	0.195	0.564 ± 0.005	0.73186 ± 15
60735	67	265	0.254	0.736 ± 0.007	0.74007 ± 8
69625	97	284	0.342	0.991 ± 0.009	0.75086 ± 14
69638	108	195	0.554	1.61 ± 0.01	0.78263 ± 5
60738	112	155	0.720	2.10 ± 0.02	0.80390 ± 11
69637	138	153	0.905	2.64 ± 0.02	0.83225 ± 9
69636	134	139	0.959	2.81 ± 0.02	0.84261 ± 8
69627	123	123	1.00	2.93 ± 0.03	0.84602 ± 12
69642	139	134	1.05	3.08 ± 0.03	0.85482 ± 14

The value of $^{87}\text{Sr}/^{86}\text{Sr}$ for the SRM 987 standard was 0.71021 ± 0.00008 normalized to a $^{88}\text{Sr}/^{86}\text{Sr}$ value of 8.3752. The value of $1.42 \times 10^{-11} \text{ yr}^{-1}$ was used for the decay constant of ^{87}Rb . All errors are quoted at the 2σ level. The Rb and Sr concentrations (in p.p.m.) and the Rb/Sr ratios were determined by X-ray fluorescence spectrometry. The concentrations are accurate to $\pm 5\%$.

* Omitted from isochron, severely epidotized.

Table 2 Samarium-neodymium analytical data, Mount Narryer

Sample	$^{147}\text{Sm}/^{144}\text{Nd}$	$^{143}\text{Nd}/^{144}\text{Nd}$	$f_{\text{Sm}/\text{Nd}}$	ϵ_{Nd}^0	$T_{\text{CHUR}}^{\text{Nd}}$ (Myr)
60734	0.07892 ± 23	0.509884 ± 30	-0.5988	-53.4	3510 ± 50
60735	0.08751 ± 26	0.509996 ± 20	-0.5551	-51.2	3630 ± 40
BCR-1	—	0.51263 ± 3	—	—	—

The samples were digested in HF/HClO₄/HCl acids in a Teflon bomb. A portion of each sample was spiked with a $^{147}\text{Sm}/^{150}\text{Nd}$ dual tracer, and the spiked and unspiked samples were separated using ion exchange procedures. Both Sm and Nd were analysed on triple rhenium filament assemblies as Nd⁺ and Sm⁺ ions. Nd isotopic ratios were measured with respect to ^{142}Nd and corrected for fractionation using $^{146}\text{Nd}/^{142}\text{Nd} = 0.632265$ (ref. 12). Evidence for Ce contamination was checked at mass 140. The uncertainties are 95% confidence limits. The analytical blank contributed <1 part in 10^4 of the total Nd. $f_{\text{Sm}/\text{Nd}}$, ϵ_{Nd}^0 and $T_{\text{CHUR}}^{\text{Nd}}$ are calculated using $(^{147}\text{Sm}/^{144}\text{Nd})_{\text{CHUR}} = 0.1967$ and $(^{143}\text{Nd}/^{144}\text{Nd})_{\text{CHUR}} = 0.51262$ (ref. 13).

These data strongly support the emerging picture in the Yilgarn Block of an ancient gneiss basement, now exposed in the west, overlain by greenstones, now exposed in the central part. Additional comparative Sm-Nd and Rb-Sr studies are planned on regional transects up to 800 km in length, across the greenstone terrains to clarify the chronological relations between the gneiss and greenstones, and to detect any regional variation in greenstone ages. Such studies may help distinguish between a model of lateral accretion of successively younger greenstones away from an embryonic craton, or a single widespread event of greenstone accumulation on a continuous basement. This may also clarify the curious situation observed in the Yilgarn Block of younger rocks occurring on the concave side of an arcuate belt of older rocks, a feature that does not fit with an island-arc accretionary model.

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Nd and Sr isotopic relationships in pelagic clays and ferromanganese deposits

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The concentrations of heavy metals in seawater are exceedingly low and commonly less than 10^{-9} g per g. In marked contrast, their concentrations in pelagic clays and ferromanganese deposits occurring on the ocean floor are many orders of magnitude greater. This relationship is a reflection of both the low solubilities of heavy metals in seawater and their efficient sequestering from seawater by particulates. The behaviour of the heavy metal neodymium in the marine environment is further investigated in this study through the useful natural isotope tracer ^{143}Nd . $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ ratios are reported for hydrogenous ferromanganese sediments, the lithic substrate of a manganese nodule and pelagic clay samples occurring close to manganese nodules. Common sources for Nd in clays, ferromanganese deposits and pelagic clays are demonstrated, but significant differences exist between the Atlantic, Indian and Pacific Oceans.

Ferromanganese sediments are broadly divisible into hydrogenous and hydrothermal deposits¹. Hydrogenous deposits are considered to form by slow ($0.1\text{--}1.0\text{ mm Myr}^{-1}$) precipitation of Fe and Mn (refs 2–4) and include ferromanganese pavements on marine topographic highs, and concretions on abyssal plains. Hydrothermal deposits are considered to form by much more rapid ($1\text{--}10\text{ mm kyr}^{-1}$) precipitation of Fe and Mn (refs 3, 5) and include basement associated ferromanganese crusts and the so-called metalliferous sediments^{6–9}.

The sources of elements incorporated into ferromanganese deposits vary according to the type of deposit and geographical location. Although the continents are usually considered to be the major source of the Fe and Mn in hydrogenous deposits, submarine volcanism may also contribute in some areas¹⁰. Even within a single deposit, the provenances of the different major, minor, and trace elements may not be the same. Whilst Fe and Mn in metalliferous sediments occurring at the East Pacific Rise are thought to be derived predominantly from oceanic basalt^{8,9,11}, the isotopic compositions of O, S, Sr and U in these deposits differ from those found in oceanic basalts and are compatible with a seawater origin^{8,9}. Further, although Pb-isotope compositions in the East Pacific Rise deposits are within the range found in mid-ocean ridge basalts^{9,12}, the range of Pb-isotope compositions in Pacific hydrogenous ferromanganese deposits encompasses many of the variations found in oceanic basalts and pelagic clays^{12–14}.

In contrast with the large variability of Pb isotope compositions, the $^{143}\text{Nd}/^{144}\text{Nd}$ ratios of all measured Pacific hydrothermal and hydrogenous ferromanganese deposits vary within

a narrow range, and are less than those measured for Recent mid-ocean ridge basalts and also less than the value for the model bulk Earth (Fig. 3a). These observations suggest that the Nd in these deposits is predominantly continent-derived.

The median $^{143}\text{Nd}/^{144}\text{Nd}$ ratio of Pacific Ocean surface ferromanganese deposits is greater than for the Indian Ocean, which is in turn greater than the median ratio for the Atlantic Ocean (Fig. 3a). In each case, the $^{143}\text{Nd}/^{144}\text{Nd}$ ratios vary within a small range (less than $\pm 2 \times 10^{-4}$), indicating that each ocean is isotopically well-mixed with respect to Nd. $^{143}\text{Nd}/^{144}\text{Nd}$ ratios in seawater samples from the Pacific and Atlantic oceans are within the range of $^{143}\text{Nd}/^{144}\text{Nd}$ ratios for ferromanganese deposits within these oceans^{15,16}. $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of all surface ferromanganese samples measured cluster around the present day seawater value (0.7091). These observations raise questions concerning the mechanisms by which different elements are transported to their sites of deposition. An understanding of such mechanisms must involve identification of the ultimate provenance of the elements, their fluxes between different reservoirs, and the degree of mixing within each reservoir. This study investigates the behaviour of Nd and Sr in the marine environment and focuses on the isotopic relationships of Nd in ferromanganese deposits and spatially related pelagic clays.

The locations of ferromanganese and oceanic clay samples analysed, together with locations of ferromanganese, pelagic clay and seawater samples previously analysed for Nd isotopes^{12,15–17}, are shown in Fig. 1. All of the Nd and Sr isotope analyses reported in Table 1 were made at the Lamont-Doherty Geological Observatory using techniques previously described^{18,19}. For those samples leached with acetic acid (HOAc), isotope data are reported for both leachates and residues (Table 1).

The $^{143}\text{Nd}/^{144}\text{Nd}$ ratios for samples of surface ferromanganese deposits from the Pacific and Indian oceans (Fig. 3, Table 1) fall within the ranges reported previously for samples from these oceans^{12,16}. Sample D-9, described elsewhere⁵, from the Galapagos spreading axis consists of a hydrothermal ferromanganese crust layer in contact with a hydrogenous ferromanganese crust. The hydrothermal crust was deposited at, or near, the ridge axis, whereas the hydrogenous crust was apparently deposited at a locale where hydrothermal effects were absent. The $^{143}\text{Nd}/^{144}\text{Nd}$ ratios obtained for these distinct layers (Table 1) are indistinguishable and fall within the narrow range found for all other ferromanganese and seawater samples from the Pacific Ocean (Fig. 3a), which range from 0.5124 to 0.5126. $^{143}\text{Nd}/^{144}\text{Nd}$ ratios in ferromanganese samples from the Atlantic and Indian oceans are significantly lower than the Pacific Ocean samples and range from about 0.5119 to 0.5122 (Fig. 3a). The median of the Atlantic Ocean samples is at a lower $^{143}\text{Nd}/^{144}\text{Nd}$ ratio than the median of Indian Ocean samples, but the larger range of values for the Atlantic Ocean samples does indicate real variability of provenance. Piepgras and Wasserburg¹⁶ have shown that measurable variability in $^{143}\text{Nd}/^{144}\text{Nd}$ ratios extends to different depths within the same water column in the Atlantic Ocean. The total range of measured Atlantic seawater values is within the range found for Atlantic ferromanganese deposits. The relative differences in the $^{143}\text{Nd}/^{144}\text{Nd}$ ratios found in the Atlantic compared with the Pacific samples are compatible with an older overall crustal source of Nd in Atlantic ferromanganese deposits compared with Pacific deposits.

As part of a general survey of pelagic clays, $^{143}\text{Nd}/^{144}\text{Nd}$ ratios are presented in Table 1 for surface clay samples from the Atlantic, Indian and Pacific oceans (Fig. 1) that are spatially related to manganese nodules. The $^{143}\text{Nd}/^{144}\text{Nd}$ ratios of Indian and Atlantic samples are within the ranges established for the ferromanganese deposits from the same ocean (Fig. 3a, b), which suggests that the provenance of Nd in both clays and ferromanganese deposits within these oceans are closely related. The reported $^{143}\text{Nd}/^{144}\text{Nd}$ ratio of 0.512423 ± 21 for an eastern Pacific Ocean clay sample¹⁷, is within the range established for Pacific Ocean ferromanganese deposits. Three clay samples

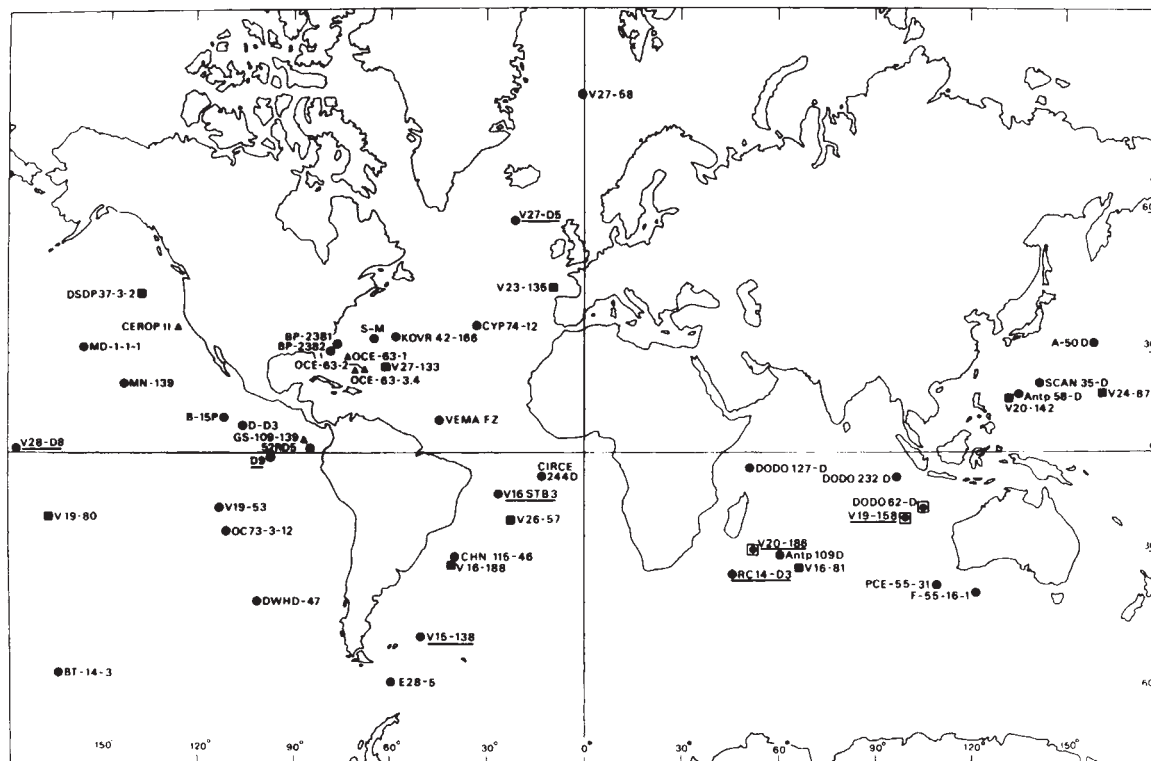


Fig. 1 Location of ferromanganese deposits (●) analysed as part of this study (underlined) and previously published^{12,15}. Seawater sites (▲) from refs 15, 16. Locations of pelagic clay samples analysed as part of this study (□, ■) and red clays (DSDP-37-3-2 and DODO-62D) from refs 17, 15 respectively.

from the western Pacific (Table 1, Fig. 3b) have $^{143}\text{Nd}/^{144}\text{Nd}$ ratios close to 0.5122, lower than those of manganese nodules sampled from similar locations, indicating a decoupling between Nd in clays and nodules. In contrast, data for the Atlantic and Indian oceans suggest that Nd in both clays and nodules may be of the same derivation. If, for example, Nd in western Pacific manganese nodules is simply considered to be a mixture of a component isotopically similar to western Pacific pelagic clays ($^{143}\text{Nd}/^{144}\text{Nd} \approx 0.5122$) and a basaltic component ($^{143}\text{Nd}/^{144}\text{Nd} \approx 0.5130$), then the latter component would comprise ~25% of the Nd in these nodules. It remains to be shown whether the higher mean seafloor spreading rate in the Pacific compared with the Atlantic and Indian oceans is the cause of these observations.

The relationship between Nd and Sr isotopes in a manganese nodule (located at a depth of 400 cm in Vema core V20-186 from the Indian Ocean) and the enclosing lutite has been examined in addition to the general survey of pelagic clays. Six samples of sediment from above and below the manganese nodule, together with two samples of the nodule itself, were leached with HOAc. Measured $^{87}\text{Sr}/^{86}\text{Sr}$ ratios for leachates and residues, and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios for the residues (Table 1, Fig. 2) demonstrate the existence of an HOAc leachable component in both the clays and the nodule with a $^{87}\text{Sr}/^{86}\text{Sr}$ ratio close to 0.709, the present day value of seawater. The $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of clay residues are greater than 0.726. Those for the nodule residues are close to 0.710, significantly higher than present day seawater, and may reflect occlusion of some clay in the nodules. Although typical concentrations of Nd in manganese nodules are nearly an order of magnitude higher than pelagic clays²⁰, the $^{143}\text{Nd}/^{144}\text{Nd}$ ratios of the lutite and manganese nodule samples are very similar with a total range of 0.511940–0.512011 (Table 1, Fig. 2), and this cannot be attributed to the inclusion of clay into the nodules. This contrasts with the greater differences in $^{87}\text{Sr}/^{86}\text{Sr}$ ratios between the manganese nodule and lutite and suggests that, in contrast to Sr, the provenance of Nd in the lutite and the nodules may be the same. $^{143}\text{Nd}/^{144}\text{Nd}$ ratios for clay and nodule samples from the top of a second Indian Ocean core

(V19-158) are 0.512128 ± 24 and 0.512214 ± 28 respectively. These results are similar to the ratios obtained for an Indian Ocean clay-nodule pair (0.512140 ± 25 and 0.512246 ± 26 respectively)¹⁵. In both cases the $^{143}\text{Nd}/^{144}\text{Nd}$ ratios of the clay and nodule samples are within the range of all Indian Ocean surface samples measured (Fig. 3a, b).

The relationships between the $^{143}\text{Nd}/^{144}\text{Nd}$ ratios in surface ferromanganese deposits and pelagic clays within each ocean suggest that the Nd budget of ferromanganese deposits may be dominated by the same source that supplies pelagic clays. Ferromanganese deposits have a minor role in the overall Nd budget of the oceans. The largest repository of Nd in the ocean basins is pelagic clay (~40 p.p.m. by weight of Nd). Although manganese nodules possess a higher concentration of Nd than pelagic clay²⁰, the ratio of the rate of Nd accumulation in manganese nodules relative to pelagic clay is estimated (Table 2) to be about 0.05. The more restricted occurrence of manganese nodules limits the ratio of total accumulation in nodules to clay

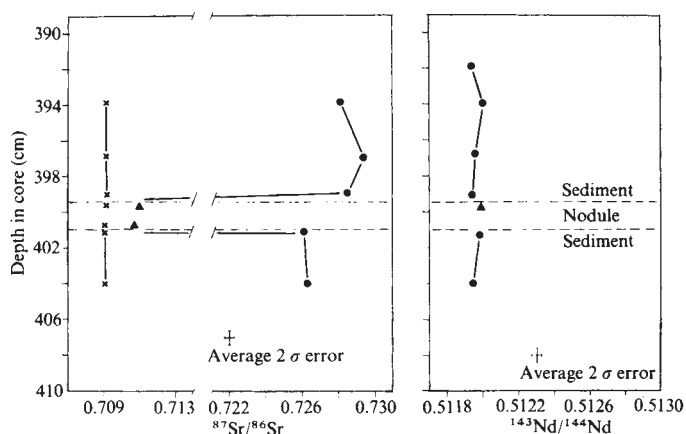


Fig. 2 $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ in core V20-186 as a function of depth. ▲, Manganese nodule; ●, sediment; ×, HOAc leachate.

Table 1 Sample locations and Nd- and Sr-isotope data

Sample	Type	Location	Depth (m)	$^{143}\text{Nd}/^{144}\text{Nd}$	$^{87}\text{Sr}/^{86}\text{Sr}^*$	$^{87}\text{Sr}/^{86}\text{Sr}^\dagger$
Pacific Ocean						
D-9†	HGC	3°02' S, 95°09' W	2,420	0.512486 ± 32	0.709184 ± 50	0.70923 ± 50
D-9	HTC	3°02' S, 95°09' W	2,420	0.512468 ± 30	0.709157 ± 50	
V28-D8	MN	0° N, 179° W	3,594–2,587	0.512465 ± 28	0.709159 ± 50	
V19-80	LUT(WR)	18°23' S, 165°44' W	4,839	0.512227 ± 30		
V20-142	LUT	17°11' N, 133°16' E	5,997	0.512251 ± 30		
V20-87	LUT	19°04' N, 161°33' E	4,819	0.512210 ± 24		
Indian Ocean						
RC14-D3	MN	34°45' S, 44°46' E	2,479–2,050	0.512142 ± 30		
V19-158	MN	18°11' S, 99°24' E	5,759	0.512214 ± 28		
V19-158	LUT(WR)	18°11' S, 99°24' E	5,759	0.512128 ± 24		
V16-81	LUT(WR)	30°38' S, 70°00' E	4,050	0.512210 ± 24		
V20-186–392 cm	LUT		5,037	0.511935 ± 30		
V20-186–394 cm	LUT			0.512011 ± 26	0.728143 ± 50	0.709203 ± 50
V20-186–397 cm	LUT			0.511947 ± 30	0.729679 ± 59	0.709225 ± 50
V20-186–399 cm	LUT			0.511940 ± 26	0.728509 ± 52	0.709204 ± 50
V20-186–400 cm	MN	28°06' S, 51°19' E		0.511996 ± 32	0.709997 ± 58	0.709180 ± 48
V20-186–401 cm	MN				0.710866 ± 50	0.709245 ± 40
V20-186–401 cm	LUT			0.511989 ± 30	0.726174 ± 50	0.709178 ± 48
V20-186–404 cm	LUT			0.511943 ± 32	0.726241 ± 50	0.709263 ± 45
Atlantic Ocean						
V16-SBT3	MN	13°04' S, 24°41' W	4,415	0.512078 ± 28	0.709244 ± 48	
V16-SBT3	SUBST.	13°04' S, 24°41' W	4,415	0.512444 ± 30	0.707420 ± 56	
V27-D5	MN	56° N, 21° W	1,641–1,509	0.512109 ± 24	0.709150 ± 34	
V15-138	MN	49°35' S, 48°05' W	2,725	0.512206 ± 32		
V26-57	LUT	20°46' S, 24°08' W	5,297	0.512150 ± 20		
V27-133	LUT(WR)	25°58' N, 60°19' W	4,682	0.512056 ± 24	0.721152 ± 50	
V23-135	LUT(WR)	45°05' N, 7°56' W	5,868	0.5119825 ± 26	0.722680 ± 46	
V16-188	LUT	30°56' S, 39°37' W	4,665	0.512241 ± 24		

MN, manganese nodule; HGC, hydrogenous crust; HTC, hydrothermal crust; LUT, lutite; SUBST, lithic substrate of nodule V16-SBT3. $^{143}\text{Nd}/^{144}\text{Nd}$ data normalized to $^{146}\text{Nd}/^{144}\text{Nd} = 0.7219$.

$^{87}\text{Sr}/^{86}\text{Sr}$ ratios expressed relative to $^{87}\text{Sr}/^{86}\text{Sr} = 0.70800$ for Eimer and Amend SrCO_3 . Measured value for this standard is 0.70806.

Reported errors are 2σ on the mean.

* $^{87}\text{Sr}/^{86}\text{Sr}$ of whole rocks (WR) or HOAc residue.

† $^{87}\text{Sr}/^{86}\text{Sr}$ of HOAc leachate.

‡ Sample D-9 described by Moore and Vogt⁵; all others from Lamont-Doherty Geological Observatory.

to <0.05 . The total mass of Nd dissolved in seawater, $\sim 4 \times 10^9$ kg using Hogdahl's²¹ estimate for the concentration of Nd in seawater (Table 2), is trivial compared with the mass of Nd in pelagic clay. For example, a mere 0.4 mm of abyssal carbonate-free sediment is estimated to contain an amount of Nd equal to the total dissolved Nd in a water column of average depth (Table 2). A much larger sediment depth maintains contact with seawater by bioturbation processes. In other words, a particulate clay concentration in seawater of $75 \mu\text{g l}^{-1}$ would possess an amount of Nd equal to the estimated average dissolved load. Note that the pelagic clay sedimentation rate estimates suggest that seawater should contain an average of $\sim 50 \mu\text{g l}^{-1}$ of particulate material, or an amount of Nd in particulate material approximately equal to the dissolved load (Table 2). Particulate concentrations are much higher than average in the nepheloid zone, which contains pelagic clay swept back up into the water column, and may extend 1,000 m above the sediment-seawater interface.

An outstanding problem is the mechanism by which the related $^{143}\text{Nd}/^{144}\text{Nd}$ ratios in ferromanganese deposits, pelagic clays and seawater are established. For example, continual cation exchange between pelagic clay and seawater, particularly within the nepheloid zone, may control the Nd isotope composition of seawater. If cation exchange is the controlling factor, then the $^{143}\text{Nd}/^{144}\text{Nd}$ ratios of western Pacific pelagic clays require that some of the Nd in the clay be isolated from chemical contact with seawater, as occurs with Sr (ref. 22). Addy²³ has interpreted rare-earth element (REE) abundance patterns in Atlantic red clays in terms of a detrital Nd component ($\sim 90\%$), and a hydrogenous component ($\sim 10\%$). The hydrogenous component must be sequestered from seawater, and may preferentially exchange with it.

Degradation of particulate material in river systems provides the initial source of marine Nd; however, the $^{143}\text{Nd}/^{144}\text{Nd}$ ratios of water and particulates in different rivers are likely to be highly

variable. Even though some 98% of the annual river flux of Nd is in particulate material^{24,25}, the estimated average concentration of dissolved Nd in river water is over an order of magnitude higher than in seawater^{24–26}. If the dissolved Nd concentration in seawater is at a steady state, the excess Nd in river water must be sequestered by particulate material in the oceans, and may constitute a portion of the Nd in the hydrogenous REE component of pelagic clays. The small but distinct range of $^{143}\text{Nd}/^{144}\text{Nd}$ ratios in ferromanganese deposits and seawater within each ocean is therefore a function of the residence time of Nd in seawater, the kinetics of any important exchange processes, and intra- and inter-ocean mixing times.

Estimates of the oceanic Nd-residence time^{27,28} ($\sim 2\text{--}3 \times 10^2$ yr) are comparable to estimates of the residence time of Pb

Table 2 Mass balances and residence time of Nd in ocean basins

Average concentration of Nd in seawater ²¹	$2.8 \times 10^{-9} \text{ g l}^{-1}$
Thickness of pelagic sediment (averaged, over oceans) containing mass of Nd equivalent to that in seawater	0.4 mm
Mass of clay particulate containing equivalent amount of Nd as 1 litre seawater	$\sim 7.5 \times 10^{-5} \text{ g}$
Average amount of non-carbonate detrital particulate in seawater	$5 \times 10^{-5} \text{ g l}^{-1}$
τ_{Nd} (residence time of Nd) assuming particulate Nd isolated from dissolved Nd	3,100 yr

Calculations assume steady state and the following: Nd concentration in pelagic clay equal to average North American shale = 37 p.p.m.; density of dry abyssal sediment = 0.7 g cm^{-3} (ref. 3); abyssal non-carbonate sedimentation rate = $10^{-4} \text{ g cm}^{-2} \text{ yr}^{-1}$ (ref. 3); mean ocean depth = 3,729 m (ref. 3); pelagic sediment $\sim 90\%$ carbonate (ref. 3); manganese accumulation rate in nodules = $10^{-6} \text{ g cm}^{-2} \text{ yr}^{-1}$ (ref. 3); Nd concentration in river water = $3.79 \times 10^{-7} \text{ g l}^{-1}$ (ref. 25); H_2O flux from rivers in oceans = $3.2 \times 10^{16} \text{ l yr}^{-1}$ (ref. 38).

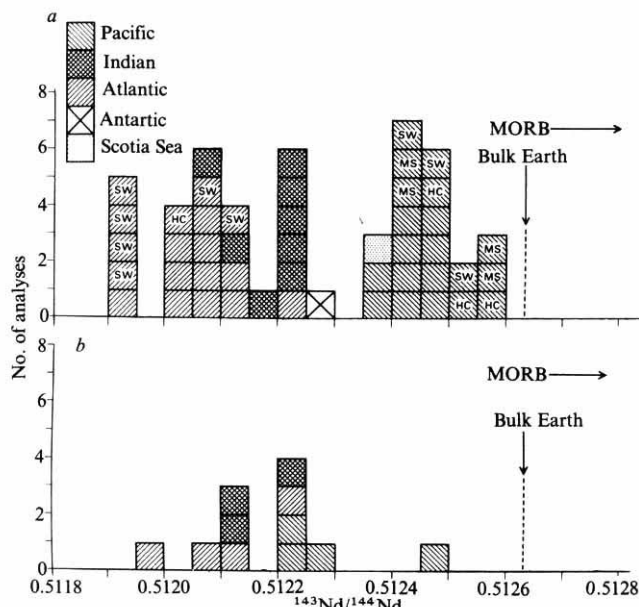


Fig. 3 *a*, Histogram of $^{143}\text{Nd}/^{144}\text{Nd}$ ratios in ferromanganese samples from this study plotted together with analyses from refs 12, 15. MS, metalliferous sediment; HC, hydrothermal crust; SW, seawater. Unlabelled boxes are manganese nodules. *b*, Histogram of pelagic clay samples from this study together with a sample from ref. 17 and one from ref. 15. All $^{143}\text{Nd}/^{144}\text{Nd}$ ratios are relative to $^{146}\text{Nd}/^{144}\text{Nd} = 0.7219$.

(refs 29–33). In these estimates the abundance term is based on the concentration of dissolved Nd in seawater, and the input term is based on the total load of Nd entering the oceans per year. The actual residence times will be greater than these minimum values if portions of the REE which enter the oceans are isolated from chemical contact with seawater, as is indicated by the western Pacific clays. If the REE within particulates are completely isolated from seawater, for example, τ_{Nd} may be as long as 3,100 yr. The intra-ocean uniformity and inter-ocean differences in the $^{143}\text{Nd}/^{144}\text{Nd}$ ratios of surface ferromanganese deposits contrasts with the intra-ocean variability of Pb isotope ratios, and implies that τ_{Nd} is greater than τ_{Pb} .

There have been various opinions on the direct source of the REE incorporated into ferromanganese deposits. Goldberg *et al.*²⁷ noting the similarities between rare-earth patterns of manganese nodules and seawater, favour direct precipitation of the REE from seawater. Their suggestion has been supported for both manganese nodules^{12,20,34,35} and East Pacific Rise metalliferous sediments^{8,9,12}. Ehrlich³⁶ and Bender³⁷, on the other hand, suggest that REE are incorporated into manganese nodules from proximal clay either by surface transfer mechanisms, or by clay occlusion into the nodule. The apparent conformability of Nd isotope ratios in Pacific seawater samples and ferromanganese deposits has led Piepgras *et al.*¹⁵ to favour seawater as the source of Nd in ferromanganese deposits. A distinction between a seawater or pelagic clay source of Nd in manganese nodules cannot be determined from the results on Atlantic and Indian ocean samples (Fig. 3*a, b*). However, the demonstrated decoupling between Nd in clay and manganese nodules in the western Pacific suggests that Nd in the manganese nodules is not representative of the total Nd in the clay. Although pelagic clay may contain both labile and non-labile Nd components, the simplest explanation of the data on hand is to invoke seawater as the direct source of the REE in manganese nodules.

$^{143}\text{Nd}/^{144}\text{Nd}$ ratios in surface pelagic clay samples from different oceans exhibit the same general relationship as those from ferromanganese deposits. That is, the median $^{143}\text{Nd}/^{144}\text{Nd}$ ratio of Pacific Ocean clays is higher than the median of Atlantic Ocean clays, which is in turn higher than the median of Atlantic Ocean clays. This result demonstrates the domination of continent derived detrital material in controlling the Nd-budget

of clays, ferromanganese deposits and seawater within each ocean basin, and highlights the differences between them.

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^{15}N abundance in Antarctica: origin of soil nitrogen and ecological implications

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Antarctica offers a unique natural environment from the point of view of nitrogen cycling. Ice-free dry areas, 'Antarctic oases', and penguin rookeries are of particular interest because of the characteristic distribution of nitrate deposits^{1–4} and biological materials such as algae and guano. As the distribution of $^{15}\text{N}/^{14}\text{N}$ in a geochemical system reflects sources and metabolic pathways of nitrogen⁵, a study of the nitrogen isotopic composition of Antarctic samples would help us to understand the Antarctic nitrogen cycle. Here we report that nitrate in Antarctic soils is extremely depleted in ^{15}N compared with biogenic nitrogen, and that algae collected from a nitrate-rich saline pond and from a penguin rookery exhibit, respectively, the lowest and the highest $^{15}\text{N}/^{14}\text{N}$ ratios among terrestrial biogenic nitrogen so far observed. We also discuss possible causes of these extreme nitrogen isotopic compositions.

Table 1 Distribution of chemical components and $\delta^{15}\text{N}$ of nitrate for soil materials from Wright Valley, Victoria Land, Antarctica

Sample	Location	Mansell colour	Texture	pH(H ₂ O)	Cl ⁻ (mequiv. per g)	NO ₃ ⁻ -N (µg atom N per g dry soil)	NO ₂ ⁻ -N (µg atom N per g dry soil)	NH ₄ ⁺ -N (µg atom N per g dry soil)	$\delta^{15}\text{N}_{\text{NO}_3^-}$ (‰)	Remarks
On northern side of Lake Vanda, 175 m height										
V-1	77°31.1' S 161°41.1' E	5Y5/2	Silty clayey loam	6.28	2.06	63.6	0.00	0.08	-23.4	Slope of a U-shaped valley
V-2	77°30.9' S 161°39.9' E	5Y6/4	Sandy loam	7.58	0.999	22.9	0.00	0.01	-22.2	In the delta
V-3	77°30.9' S 161°38.4' E	7.5Y6/2	Loam	7.28	0.681	19.3	0.00	0.00	-20.6	In the delta, containing white evaporites
Near Bull Pass										
P-1	77°31.3' S 161°49.8' E	7.5Y7/2	Silty loam	7.89	0.479	37.9	0.00	0.00	-18.3	Above pecten fossil layer
P-2	77°31.3' S 161°49.8' E	5Y6/4	Sand	8.10	0.191	58.6	0.00	0.04	-18.6	Below pecten fossil layer
On southern side of Lake Vanda										
2	77°32.7' S 161°31.8' E	5Y8/3	Loam	8.59	0.346	10.0	0.00	0.00	-11.5	
3	77°32.7' S 161°31.8' E	2.5Y6/4	Sandy loam	8.51	0.095	10.0	0.00	0.00	-16.1	
4	77°32.7' S 161°31.8' E	2.5Y6/4	Sandy loam	8.02	0.100	53.6	0.00	0.00	-14.8	
6	77°32.7' S 161°31.8' E	10YR4/4	Loam	7.46	0.994	179	0.00	0.00	-14.1	

Table 2 Distribution of $\delta^{15}\text{N}$ in various nitrogenous substances from Wright Valley, Victoria Land and Ross Island, Antarctica

Sample	Location	$\delta^{15}\text{N}$ (‰)	Remarks
Epibenthic algae			
V-5	77°31.7' S 161°38.6' E	-9.0	Southern shore of Lake Vanda, a saline and meromictic lake
L-1	77°32.4' S 160°45.2' E	-47.8	Unnamed saline pond, Labyrinth, near the edge of the Wright Glacier
		-49.0	
C-2	77°32.8' S 161°31.4' E	+4.8	Eastern shore of Lake Canopus, a freshwater lake
B-1	77°31.5' S 161°42.2' E	-9.1	Lake Bull, a freshwater lake
		-9.3	
Algal felt			
R-1	77°12.3' S 166°28' E	+30.7	Northern penguin rookery at Cape Bird, Ross Island
Organic nitrogen			
Soil	77°12.3' S 166°28' E	-28.6	Northern penguin rookery at Cape Bird, Ross Island Mansell colour, 7.5YR 1.7/1; texture, sand; pH(H ₂ O), 7.6; Cl, 0.032 mequiv. g ⁻¹
Sediment	77°32.1' S 161°32.2' E	-7.3	Bottom sediment of Lake Vanda depth, 67.5 m

'The Dry Valleys' in southern Victoria Land, McMurdo Sound region is a large polar desert extending over an area of ~2,500 km² and containing several valley systems—Wright Valley being the most famous. Precipitation is <100 mm yr⁻¹ and mean air temperature is between -18 and -25 °C. Fallen snow is mostly dissipated by sublimation, but melting of glaciers occurs during the warm season and produces fresh and saline lakes and ponds where algal communities flourish. According to Webb⁶, the Wright Valley was below sea level in the Pliocene (3–4 Myr BP).

Soil materials and algal samples were collected from Wright Valley and Cape Bird in Ross Island in January 1979. Procedures for isotopic analysis were described elsewhere^{7,8}. Nitrogen isotopic composition is expressed as follows:

$$\delta^{15}\text{N}(\text{‰}) = \left\{ \frac{(^{15}\text{N}/^{14}\text{N})_{\text{sample}}}{(^{15}\text{N}/^{14}\text{N})_{\text{air}}} - 1 \right\} \times 1,000$$

Water soluble salts were analysed by the following methods: for ammonium, phenol-hypochlorite method⁹; for nitrate, diazotization method¹⁰; for nitrate, cadmium reduction method¹¹; and for chloride, mercuric thiocyanate method¹². Organic nitrogen was determined by Kjeldahl digestion¹³.

Nitrate was the major component among nitrogenous compounds in Antarctic soils (Table 1). Concentrations of organic nitrogen (0.34–6.4 µg atom N per g dry soil) were one to two orders of magnitude lower than those of nitrate except for samples collected from a penguin rookery, Ross Island. Our data of $\delta^{15}\text{N}$ for soil nitrate in Antarctica exhibited quite low values (-11 to -24‰). The minimum value of -23.4‰ was found for soil materials collected from a slope of a U-shaped valley near Lake Vanda. The natural abundance of ^{15}N in biogenic nitrogen in the literature generally ranges from -17 to +20‰. The average $\delta^{15}\text{N}$ values for nitrate are: 3.2‰ for forest soils; 6.4‰ for cultivated soils; 7.0‰ for oceanic waters; and

-6.6‰ for rain waters^{13–15}. The $\delta^{15}\text{N}$ values for Antarctic nitrate were, thus, quite different from that for oceanic nitrate, and gave the lowest ranges among terrestrial nitrate.

Various origins have been considered for the salts in the Dry Valleys: direct marine incursion¹⁶, marine aerosols or sea spray³, chemical weathering of minerals¹⁷, and NO_x produced by auroral activities¹⁸. According to Moore¹⁹, gaseous ammonia (-10.0‰) and nitrogen dioxide (-9.3‰) in clean air are depleted in ^{15}N as compared with those in aerosols (NH₄⁺, +5.6‰ and NO₃⁻, 5.0‰). He interpreted the low ^{15}N content in gaseous compounds as being due to the nitrogen isotope fractionation associated with isotopic equilibria between gaseous and condensed phases with subsequent removal of the condensed phase. The successive removal of the condensed phase during long distance transportation from the tropical and temperate region to the polar region may further enhance depletion of ^{15}N in nitrogenous compounds in the atmosphere of the polar region. The $\delta^{15}\text{N}$ data suggest that nitrate in Antarctica soils is mainly derived from atmospheric precipitation which carries NO_x depleted in ^{15}N . NO_x depleted in ^{15}N might be produced by photochemical reactions³ as well as by auroral activities¹⁸. No other processes can produce nitrogen oxides of such low $\delta^{15}\text{N}$.

In the case of algae, $\delta^{15}\text{N}$ values ranged between -49 and +30‰ (Table 2). The lowest value (-49‰) was found for the algae collected from a small saline pond located near the edge of Wright Upper Glacier. This is the lowest $\delta^{15}\text{N}$ value ever reported for terrestrial samples. It has been demonstrated that the isotope fractionation during nitrate assimilation by a marine diatom, *Phaeodactylum tricornutum*, is inversely related to its growth rate at low light intensities and a high nitrate concentration (10 mg atom NO₃⁻-N l⁻¹). A fractionation factor as high as 1.03 has been obtained as an extreme. A characteristic feature of Antarctic saline ponds is the high concentration of nitrogenous compounds²¹. In fact, the concentration of nitrate

in this pond was 8.7 mg atom $\text{NO}_3^- \text{N l}^{-1}$. Growth of the epibenthic algae at high nitrate concentration and low light intensity may thus be accompanied by a large nitrogen isotope fractionation. The extremely low $\delta^{15}\text{N}$ values for the epibenthic algae can thus be explained by the combination of low $\delta^{15}\text{N}$ for source nitrate and large isotope fractionation associated with nitrate assimilation.

High ^{15}N enrichments of +30.7 and +28.6‰ were found in algal and organic nitrogen collected from the Adelie penguin rookery at Ross Island, respectively (Table 2). The enrichment of ^{15}N along a food chain has been reported elsewhere²². The $\delta^{15}\text{N}$ value for marine plankton ranges from 2 to 5‰ and for fishes from 15 to 20‰ (ref. 22). Adelie penguins at Cape Bird feed on krill and fish, and deposit faecal materials including uric acid in the soils of the rookery. Soil microorganisms convert uric

acid and other organic nitrogen to ammonia which evaporates with a high nitrogen isotope fractionation. This may leave nitrogenous materials rich in ^{15}N in soils. Kreitler⁷ obtained an apparent fractionation factor of 1.038 for NH_3 volatilization from barnyard soils. In the cold climate of Antarctica, the biogeochemical processes in question might proceed at an extremely slow rate. If so, a much greater fractionation of the nitrogen isotope is expected, and the high ^{15}N enrichments in organic nitrogen and algae in the penguin rookery can be explained.

Torii *et al.*²¹ observed a high pH of 9.4 in the surface water of Lake Canopus where intensive photosynthesis occurred. The isotope fractionation associated with ammonia volatilization thus also explains ^{15}N enrichment of epibenthic algae in this lake (C-2 in Table 2).

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Chemical flux in an acid-stressed stream

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The acidity of rain and snow falling on widely separated areas of the world has been increasing during the past 30 yr (refs 1–3). Acid rainfall consists of a dilute solution of sulphuric and nitric acids due to the oxidation and hydrolysis of airborne sulphur and nitrogen⁴ and frequently has a pH of <4.0. Recent studies have shown that acid rain alters the chemistry and biology of streams and lakes in large regions of the world^{1,3}. Results from reconnaissance studies in the field^{5–9} and physiological studies in the laboratory⁹ show that diversity and numbers of aquatic organisms of all major trophic levels are affected by low pH (high acidity). The quantitative effects of such acidification on biogeochemistry and biological function in natural streams have received little attention. In contrast, much is known about aquatic ecosystems affected by acid mine drainage^{10–13}. However, waters receiving acid mine drainage show effects caused by metal contamination and deposition of iron oxide particulates, as well as acidification. In our study, we experimentally acidified a third-order section of a small mountain stream in the Hubbard Brook Experimental Forest, West Thornton, New Hampshire, USA. Our aim was to measure the effects of increased acidity on chemical and biological export in the stream. It was found that experimental stream acidification to pH 4 did alter the chemical and biological flux. The most significant inorganic component affected by the experiment was aluminium. A significant net flux of carbon and nitrogen occurred in the biologically bound forms but not in dissolved substances. Net flux of phosphorus was significant in biologically bound forms. The increased loss of nutrients in the particulate organic fraction was also important, particularly if scaled to the total stream ecosystem.

Areas affected by acid precipitation are often located in northern latitudes where snow accumulates during long, cold winters. Snowmelt in spring then releases a large quantity of water and ions to streams and lakes^{14,15}. For example, on average, 54% of the annual streamwater discharge within the Hubbard Brook Experimental Forest occurs between March and May, with as much as 30% occurring in April¹⁵. It has been reported that when snow thaws, the first portions of the melt water may be very acidic¹⁶. Thus in such areas, late winter or early spring thaws may cause sudden decreases in pH in aquatic ecosystems. During such snowmelt periods, pH values as low as 4.0 have been recorded in streams in Norway¹⁷, in the Adirondack Mountains of New York State¹⁸, and in the Hubbard Brook Experimental Forest, New Hampshire (N. Johnson, personal communication). Trout deaths have been observed in Norwegian streams during flushes of acidic melt water¹⁷.

Our study was done at Norris Brook, a tributary of Hubbard Brook^{19–21}. This stream flows through deciduous forest underlain by granitic bedrock and is subject to inputs of sulphuric and nitric acids in rain and snow at an annual volume-weighted pH near 4 (range 2.85 (ref. 22)–5.95 (ref. 15) for individual storms). The annual pH of Norris Brook in the study area normally ranges from 5.4 to 6.4. We experimentally decreased the pH of a third-order section of the stream to 4 (range 3.9–4.2) by continuously adding sulphuric acid (0.05–0.5M) from 18 April to 22 September 1977. The experimental design and biological effects for the entire study are detailed elsewhere^{19–21}. Sampling stations for the reference area (A) and the treatment area (B) were located 1 m above and 15 m below the acid addition point, respectively. The treatment reach of the stream between points A and B was $26.9 \text{ m}^2 \pm 0.6$ ($\bar{x} \pm \text{s.e.}$, $n = 3$) in area. The effects of hydrogen ion stress on the biogeochemistry of the stream are described here for the first 30 days (18 April to 17 May 1977). We separated our results into the first 5 days ('acute response') and the remaining 25 days ('chronic response') of hydrogen ion stress. A mass-balance approach was used in which the inputs to the treatment area minus the outputs yielded a net flux of chemicals from the area during the experimental period.

Methods for collection of water samples and chemical analysis are detailed elsewhere²⁰. For fine particulate organic matter (FPOM) measurements, aliquots of stream water were filtered through ashed 0.45- μm glass fibre filters. Filters were dried for ~2 days at 60 °C. Carbon concentrations of the particulate

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Table 1 Mean daily biomass flux of drifting invertebrates and coarse particulate organic matter (CPOM), and flux of fine particulate organic matter (FPOM) and selected dissolved substances (Ca, Al, N, organic carbon) during 5-day (acute response) and 25-day (chronic response) periods of increased hydrogen ion stress

	Acute response			Chronic response		
	Site A	Site B	% Loss or gain	Site A	Site B	% Loss or gain
Invertebrates	0.065 ± 0.011	0.025 ± 0.065	-285	0.057 ± 0.011	0.09 ± 0.017	-58
CPOM	5.5 ± 0.53	11.4 ± 1.6	-107	11.56 ± 5.89	9.49 ± 4.3	+18
FPOM	715 ± 45.9	700 ± 66.4	+2	1,320 ± 575	1400 ± 590	-6
DOC	1,400 ± 198	1,400 ± 198	0	2,000 ± 759	2,000 ± 749	0
Al	10.4 ± 0.89	27 ± 2.9	-160	70 ± 29	130 ± 51.4	-86
Ca	1,700 ± 22.4	1,700 ± 161	0	2,000 ± 1,300	2,000 ± 1,400	0
N	<8.2 ± 0.72	<8.2 ± 0.72	0	<15 ± 7.33	<15 ± 7.33	0

Site A was in the reference area and just upstream of the treatment area; site B was at the downstream end of the treatment section. The durations of the acute and chronic responses were determined by a dose-response curve of drift density (biomass per volume of water). For the first 5 days of depressed pH, the density of invertebrate drift leaving the acidified zone exceeded that entering by a factor of 1.7–3.3, with peak drift appearing only after 2 days of acid addition. For the remaining 25 days, drift density at the treatment site was 1.6-fold greater than at the reference site. The mean daily ($\pm$ s.e., $n = 5$) streamwater concentrations at site A for Ca, Al, N and DOC during the 5-day period were 1.65 ± 0.012 , 0.01 ± 0 , <0.01 , and $1.59 \text{ mg l}^{-1} \pm 0.304$, respectively; at site B the concentrations were 1.65 ± 0.013 , 0.026 ± 0.0024 , <0.01 , and $1.59 \text{ mg l}^{-1} \pm 0.305$, respectively. The site A water concentrations ($\bar{x} \pm$ s.e., $n = 7$) during the 25-day period were 1.71 ± 0.026 , 0.05 ± 0.0075 , <0.01 , and $1.99 \text{ mg l}^{-1} \pm 0.1758$, respectively, for Ca, Al, N and DOC; at site B the values were 1.71 ± 0.026 , 0.08 ± 0.0065 , <0.01 , and $1.99 \text{ mg l}^{-1} \pm 0.1843$, respectively.

matter were determined by conversion to CO_2 and IR gas analysis²³. A conversion factor of 2.22 (ref. 24) was used to change fine particulate organic carbon to FPOM.

Drift nets were positioned upstream and downstream of the acid addition point²⁰. Invertebrates and coarse particulate organic matter (CPOM) collected in drift nets were dried to a constant weight at 60°C. CPOM is defined as all materials (conifer and deciduous leaves, very small branches, and other organic particulates) retained in the 253- μm mesh drift nets. Chemical analyses of the invertebrates and organic matter were done using a conductive Jarrell-Ash (model 975) Multi-Channel Plasma Atom-COMP/Flame Emission spectrophotometer and a Perkin-Elmer (model 303) spectrophotometer, respectively. The coefficient of variation for chemical concentrations in invertebrates and organic matter ranged from 5 to 28% for all samples. We determined the net flux (A–B) of invertebrate, particulate (CPOM, FPOM), and dissolved (Al, Ca, N and organic carbon) transport modes operating in our experimental area (site B) relative to the reference section (site A). Biomass flux of invertebrates and particulate (CPOM and FPOM) matter is the product of chemical concentration (mg per g) and mass of biota collected per unit time (g day^{-1}), whereas streamwater flux (dissolved ions) is the product of chemical concentration (mg l^{-1}) and discharge of water (l day^{-1}).

A comparison of some of the major biotic and abiotic components between the upstream and downstream sites yields information on acute and chronic effects of hydrogen ion stress (Table 1). The biomass of exported invertebrates and concentration of dissolved Al were higher in the acidified area (site B), with the per cent net loss [$100(\text{input} - \text{export})/(\text{input}) = \text{net ecosystem flux}$] being greater during the first 5 days than during the remaining 25-day period (negative values equal loss and positive values equal gain between A and B, Table 1). For example, a 285% (Students' t , one-tailed, $P < 0.025$) and 58% ($P < 0.025$) net loss of invertebrate biomass from the acidified zone (26.9 m^2 study area) occurred during the 5-day (acute) and 25-day (chronic) periods, respectively; a 160% ($P < 0.005$) and 86% ($P < 0.05$) net loss of dissolved Al occurred during these same two periods. Although the net export of CPOM increased significantly (107%, $P < 0.01$) after just 5 days of acidification, the net flux of FPOM ($P > 0.25$) and dissolved Ca, N and organic carbon did not change (Table 1). Thus, increased acidity augmented the export of biomass and some dissolved chemicals to downstream reaches with a higher rate of loss occurring immediately after acidification.

Concentrations of Al, Ca, C, N, P and S in invertebrate biomass and CPOM were determined (Table 2), and the net flux (concentration $\times$ mass per unit time) of each element between site A and B is shown in Fig. 1a (acute period) and b (chronic

period). The net flux (A–B) of these elements in the dissolved fraction (from Table 1) is also included in Fig. 1. A significant increase in net flux of Al, Ca, C, N, P and S in invertebrate biomass ($P < 0.01$) occurred for the 30-day period (Fig. 1a, b). A significant net flux of these six elements in the CPOM fraction occurred during the 5-day study period ($P < 0.01$, Fig. 1a) but not during the subsequent 25-day period ($P > 0.1$, Fig. 1b).

The increased drift of invertebrates may have been due to the increased hydrogen ion concentrations, or to increased aluminum concentrations, or to a combination of both. Schofield¹⁸ has shown that deaths of brook trout in laboratory experiments occur in dilute solutions with a pH of 4.5–5.2 and containing 0.2–1.0 mg l^{-1} aluminum, whereas brook trout survive in pH 4.5–5.0 with $<0.1 \text{ mg l}^{-1}$ aluminum. The invertebrates in our study may have been affected in a similar manner^{19–21}.

Although loss of chemicals in invertebrate and CPOM fractions was significantly increased with elevated acidity, the total amounts of Al, Ca, C and N in those fractions were small compared with the amounts of these ions in the dissolved form (Table 1). However, if an assessment had been made of chemicals in the biomass of trout, sculpins, salamanders and frogs that left the experimental section during acidification, the estimates of Al, Ca, C, N, P and N lost in the biotic component would have been considerably higher. Thus the loss of biologically bound nutrients such as C, N and P is at a level that could be significant to the stream ecosystem as a whole during acute hydrogen ion stress, as these nutrients would no longer be available for use in the stressed area.

One surprising effect of higher stream acidity was the increased flux of CPOM during the first 5 days (Fig. 1a). The increased loss of CPOM may be due to: (1) a decrease in the number of collector organisms²⁵ (filter- and deposit-feeders)

Table 2 Chemical content of aquatic insects and coarse particulate organic matter collected from Hubbard Brook streams

Chemical	$\bar{x}$ (mg per g dry mass $\pm$ s.e.)	
	Aquatic insects ($n = 7$)*	CPOM ($n = 5$)†
C	473 $\pm$ 1.1	213 $\pm$ 35.2
N	75 $\pm$ 3.0	10 $\pm$ 2.0
P	9.1 $\pm$ 0.6	0.64 $\pm$ 0.13
S	5.4 $\pm$ 0.49	0.66 $\pm$ 0.18
Ca	2.0 $\pm$ 0.4	1.3 $\pm$ 0.30
Al	0.84 $\pm$ 0.14	8.2 $\pm$ 0.70
C:N:S:P	52:8.2:0.59:1	333:15.6:1.03:1

* Norris Brook, this study.

† Headwater streams in the Hubbard Brook Experimental Forest (R. Bilby, personal communication).

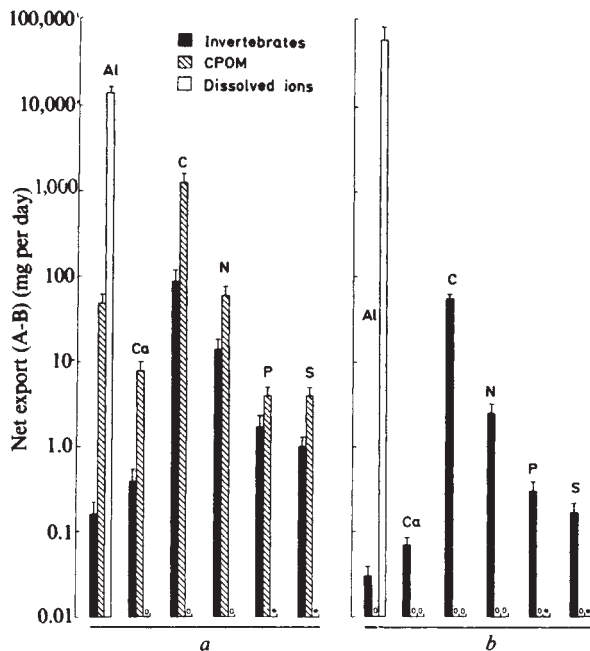


Fig. 1 Mean daily *a*, acute (5-day) and *b*, chronic (25-day) effects of elevated hydrogen ion concentration on flux of Al, Ca, C, N, P and S in invertebrates (■), CPOM (▨) and dissolved components in Norris Brook (□). Zero designates no change in net flux at site B relative to A. Vertical bars represent standard error. A greater mass was exported from B than A, thus A - B is a negative number. Asterisks indicate that flux of dissolved sulphur and phosphorus, due to elevated acidity, could not be evaluated for the 1977 study; that is, concentrated H₂SO₄ was added to the stream water to lower the pH and the quality of the dissolved phosphorus data was poor. However, based on preliminary results from an experiment in which HCl was used to lower the pH, no change in dissolved sulphate concentration was detected above and below the experimental addition, and total phosphorus was mobilized in the stream water in the experimental area relative to the reference section.

that use particulate organic matter as food and store it as biomass. In the acidified reach we observed an increase in export of collector and scraper organisms²⁰. Scrapers ingest relatively more periphyton than organic detritus in natural streams^{20,26,27}. However, with a decrease in the number of collector organisms, one would also expect an increased loss of FPOM, but the expected loss of FPOM did not occur with the exception of a 25% increase in the net flux of FPOM during the first 3 h after acidification. In addition, we suspect that collectors filter such a small portion of the total water column that increases in FPOM net flux due to decreased filtering (fewer collectors present) may not be detectable. (2) Increased hydrogen ion stress may have caused organisms to move quickly off the bottom or out of accumulations of leaf debris, thus dislodging organic matter and allowing it to be transported downstream by water currents. Based on our quantitative data we believe that the latter factor is more important.

The acute (5-day) results of our acidification experiment may approximate what happens in streams during spring snowmelt in the northeastern US. Hydrogen ions are thought to migrate to the perimeter of snow crystals during the melt-refreeze cycle^{16,28,29}. It has been reported that as much as 80% of the acidic contaminants in the snowpack may be released in the first 30% of snowmelt²⁹. Depending on rate of melt, soil frost, and other environmental conditions, when an acid-stratified snowpack begins to thaw, these hydrogen ions enter streams and may depress the pH of the water substantially. As the snowmelt period is brief^{16,17,28-30}, and because neutralization of hydrogen ions takes place rapidly due to aluminum dissolution reactions in the soil^{31,32} and the streambed (our study) and cation exchange on clay minerals³³⁻³⁵, the period of high acidity may be relatively short and/or limited to headwater tributaries. Our results show, however, that a significant increase in net flux of chemical

components, incorporated in the biomass of organisms and organic matter, occurred within the first 5 days of stream acidification.

The 25-day response of the biota to H⁺ stress may simulate results from chronic acidification from acid rain. An important repercussion of acid precipitation is the elimination of sensitive aquatic organisms²⁰. The reduction in numbers of biotic communities may have important consequences for the budget of organic matter in the headwaters of mountain streams. A greater than normal proportion of detrital material may be used by the decomposer food chain, or transported downstream, or both if detritivores had ingested and temporarily assimilated this organic matter. The altered storage, processing, and export of litter and nutrients may have either beneficial or detrimental effects on the downstream macrobenthos that otherwise would not have been affected directly by upstream acidification.

Because increased burning of coal and other fossil fuels may intensify acid precipitation and deposition in snowpacks, and because low pH values during initial snowmelt periods occur in first-, second- and third-order streams¹⁷, the loss of nutrients, in particular phosphorus and nitrogen, and energy-abundant organic detritus may be of great ecological significance in headwater mountain streams.

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¹³C evidence for dietary habits of prehistoric man in Denmark

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Carbon isotopes are fractionated by natural processes such as photosynthetic assimilation of CO₂ and its absorption in water. Carbon fractionation is affected by the type of metabolism used by the plant to fix CO₂ and differs in marine and terrestrial plants. This natural fractionation pattern is passed down the food chain and may therefore be used to elucidate questions on the origin of carbon compounds in nature. Here, the ¹³C fractionation pattern has been used to investigate the dietary habits of prehistoric man in northwestern Europe. The results show that whereas Danish Mesolithic man lived on a diet dominated by sea food, in the Neolithic the human diet consisted predominantly of terrestrial food.

Isotopic fractionation in natural carbonaceous materials relevant to prehistory has recently been analysed^{1,2} and is shown in Fig. 1 as $\delta^{13}\text{C}$ values relative to the PDB standard. Due to kinetic effects, terrestrial plants that follow the normal Calvin (C₃) photosynthesis are depleted in the heavy carbon isotopes, as shown by a change in $\delta^{13}\text{C}$ values from -6 to -8‰ in atmospheric CO₂ to ~-22 to -30‰ in terrestrial plants³. Terrestrial animals feeding on such plants show a similar ¹³C content, although with a slight shift towards higher $\delta^{13}\text{C}$ values, partly because the respired CO₂ may be enriched in the light isotopes⁴. The magnitude of the fractionation effect in animals may therefore vary both with the nature of the foodstuff and with the position in the natural food chains.

Absorption of CO₂ in water and the subsequent formation of bicarbonate are governed by kinetic effects and by thermodynamic equilibrium processes which lead to an enrichment in the heavy carbon isotopes to $\delta^{13}\text{C}$ values close to 0‰ PDB (Fig. 1). When marine bicarbonate is assimilated during photosynthesis of submerged plants, reaction kinetics again result in a depletion in the heavy isotopes, in this case to $\delta^{13}\text{C}$ values of about -10 to -18‰ and this fractionation is also reflected in marine animals.

This simple isotopic separation between terrestrial and marine plants and animals is partly obscured if Hatch-Slack photosynthesis (C₄) has dominated in the terrestrial plants. Hatch-Slack photosynthesis occurs in several plants growing in arid and subtropical regions, especially in grasses of the family Panicoideae, and leads to $\delta^{13}\text{C}$ values of between -10 and -18‰ (ref. 3) (hatched boxes in Fig. 1). The most important cultivated C₄ plants are maize, sugar cane and millet. However, if as in the present study, which concerns temperate northwestern Europe, the influence of C₄ plants can be excluded, the ¹³C fractionation pattern may be used to distinguish between a predominant reliance by man on sea food and terrestrial food^{5,6}.

Figure 2 shows the ¹³C content of organic remains of bone collagen or tissue from 28 humans living from 5200 BC to AD 1750, together with ¹⁴C dates of the samples, calibrated according to Clark⁷. Collagen from the bones was isolated as a gelatine by the method by Longin⁸, and the gelatine was converted to CO₂ which was used partly for the ¹⁴C determination in a proportional counter and partly for the ¹³C determination by a mass spectrometric analysis with a reproducibility better than 0.3‰ and an overall uncertainty of about 0.7‰. Samples of human tissues were thoroughly extracted with acids and alkalis to remove extraneous carbon compounds before conversion to CO₂. It was checked that the chemical procedures applied to collagen and tissue samples had only a negligible effect on the isotopic compositions^{6,9}.

The series also included bones of three Eskimos from Greenland whose feeding habits are known or may be safely deduced. Two of these lived at Angmagssalik, East Greenland, before

contacts with Europeans, and were therefore forced to subsist almost entirely on sea foods. This is substantiated by the difference between their apparent ¹⁴C age and their historical age, which suggests a 90–95% intake of sea food^{5,6}. The third Eskimo sample was from West Greenland and the difference in ¹⁴C content of human tissue and a reindeer fur, found together, suggests a dietary supply of sea food of about 70% (ref. 6).

The measurements on humans from Denmark proper show a clear distinction between the ¹³C content of humans from Mesolithic time and from the Neolithic and later periods. All six samples from the Danish Mesolithic had a ¹³C content similar to that of the Eskimos from historic times, and thus must represent people with a predominant reliance on sea food, comparable with that in Greenland.

This conclusion is at variance with common archaeological estimates for the period, based on bones and shells found in Mesolithic dwelling places in Denmark. However, fish bones are small and fragile and may easily have been overlooked or under-represented in the early excavations of Mesolithic sites. This is also suggested by a modern excavation of coastal Mesolithic dwelling places at Vedbæk, North Zealand, from where three of the Mesolithic skeletons originate. By a careful sieving of all the excavated materials, several thousand fragments of fish bones were recovered per square metre of the thin cultural layers (ref. 10 and K. Aaris-Sørensen, personal communication). In addition to sea food, high $\delta^{13}\text{C}$ values in humans may indicate a consumption of fish and birds from streams and lacustrine waters. When comparisons are made with archaeological estimates, it should be remembered that faunal remains from dwelling places often represent consumption during a short or seasonal stay only, whereas the ¹³C content of human bones gives an integrated measure of the composition of the mean food intake over an extended period, probably of the order of 5–10 yr.

All Mesolithic sites shown in Fig. 2 are coastal sites. A strong reliance on sea food therefore seems natural. However, all eight Neolithic skeletons represented in Fig. 2 also originate from sites or graves situated either directly on the coast or within 100–200 m of the sea. These Neolithic people were thus coast dwellers, as were the Mesolithic people, but they did not utilize the resources of the sea to the same extent as did the Mesolithic people. This suggests that a highly developed Neolithic cultural complex, with a different method of subsistence and therefore different food habits, rapidly succeeded a Mesolithic economy.

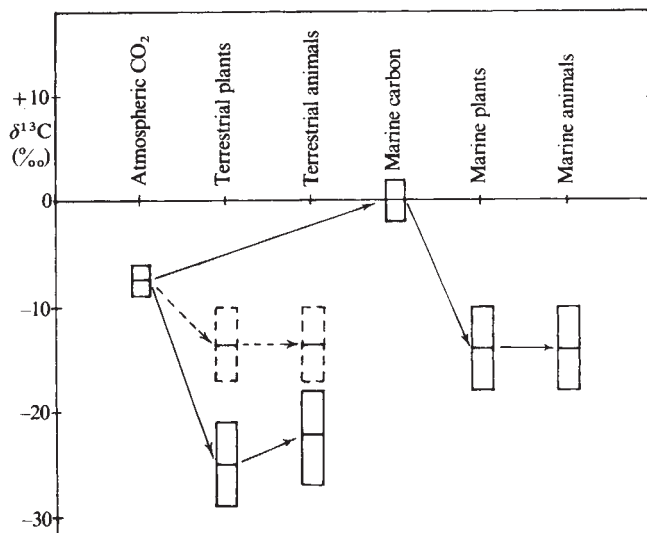
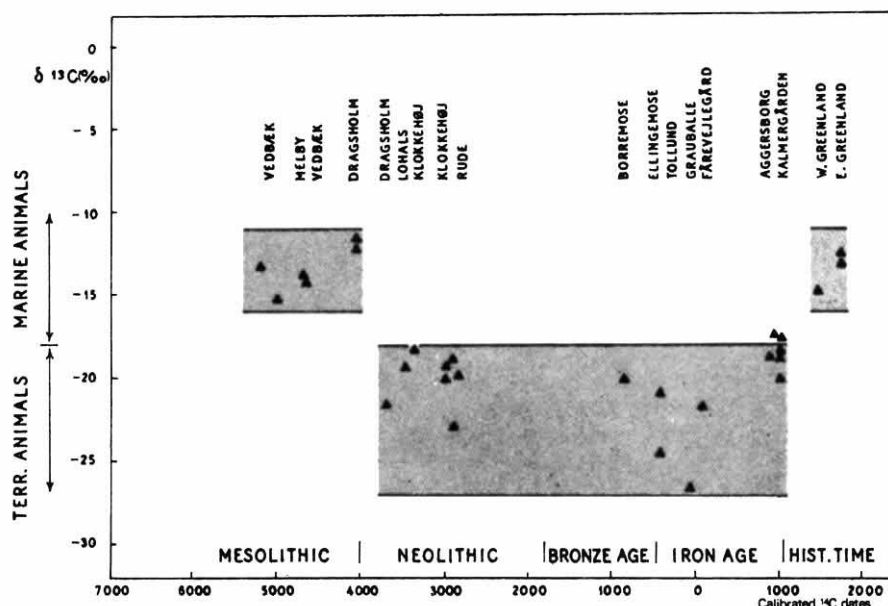


Fig. 1 Fractionation of carbon isotopes in nature. Variations in the ¹³C content are measured as

$$\delta^{13}\text{C} = \left(\frac{{}^{13}\text{C}/{}^{12}\text{C}_{\text{sample}}}{{}^{13}\text{C}/{}^{12}\text{C}_{\text{standard}}} - 1 \right) \times 1,000\%$$

with the Chicago sample of Cretaceous belemnite from the Peedee formation, South Carolina, as a standard.

Fig. 2 $\delta^{13}\text{C}$ content of human beings from Denmark and Greenland measured relative to the PDB standard versus calibrated ^{14}C dates of the samples.



With a natural scatter in the $\delta^{13}\text{C}$ values as indicated in Fig. 1, estimates of the relative proportions of dietary components from $\delta^{13}\text{C}$ measurements are rather unreliable. It is also uncertain whether part of the isotopic fractionation is influenced by species-related or individual differences in the enzymatic processes during metabolism. It is therefore only concluded that the individuals analysed from the Danish Mesolithic subsisted on a diet dominated by sea food, while those analysed from the Neolithic and later periods lived predominantly on terrestrial food, although in some cases with a significant contribution of marine food.

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The number of individuals investigated is rather small, and one should therefore be cautious in drawing far-reaching conclusions about Mesolithic food habits, but the present investigation demonstrates the potential of such measurements for the elucidation of the dietary habits of prehistoric man.

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Carbon isotope analysis of separate chemical phases in modern and fossil bone

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Studies evaluating the distribution of stable carbon isotopes in bone and other tissues of both human and non-human animals have recently been reported^{1–6}. Those investigations which examined the isotopic composition of bone have concentrated on the analysis of bone collagen and demonstrated that the $^{13}\text{C}/^{12}\text{C}$ ratios in bone collagen are directly related to the $^{13}\text{C}/^{12}\text{C}$ ratios of primary photosynthesizing plants in the diets of the animals concerned^{6–8}. With regard to archaeological applications, such analyses have been limited to relatively young samples because of the instability of collagen in bone. We have extended the isotopic method of dietary analysis by using both the organic and inorganic phases of bone with equally good results. In the case of material over a few thousand years old, unless special conditions have preserved collagen, analysis of the organic phase of bone is no longer practical due to its deterioration⁹. The technique reported here allows dietary analysis of bone over 10,000 years old by using the inorganic phase, which is more stable in fossil material.

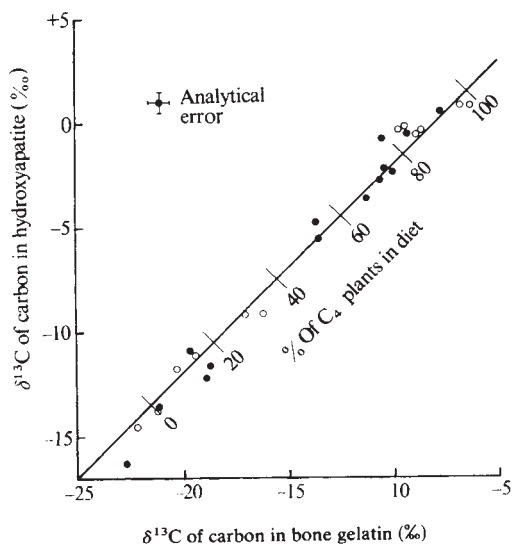
Dietary analysis using carbon isotopes is based on the fact that most plants photosynthesize by one of two pathways, the Calvin (C_3) cycle or the Hatch–Slack (C_4) pathway^{10,11}. These pathways produce very different carbon isotope ratios^{12,13} which can be readily quantified by standard mass spectrometric techniques. The carbon isotope ratios of animal tissues reflect the proportions of C_3 and C_4 plants in the animal's diet. Fresh modern bone contains at least three distinct carbon-bearing phases¹⁴. Most carbon is in the form of collagen, a complex polypeptide which represents ~10–15% by weight of fresh bone. Hydroxyapatite, also called 'bone apatite', comprises 80–90% by weight of typical bone samples. It contains a small amount of carbon, probably as carbonate ion substitutions for hydroxyl or phosphate ions. A third phase, also inorganic in nature, behaves chemically like a normal carbonate.

The main phases of bone can be separated for isotopic study by appropriate techniques. The 'normal carbonate' phase can be removed and its carbon collected as CO_2 , by reaction with cold dilute acetic acid under vacuum, leaving the insoluble hydroxyapatite and collagen. After washing to remove acetic acid residues, this material can then be reacted with dilute HCl under vacuum to hydrolyse hydroxyapatite and release its carbon as CO_2 for analysis. The collagen remains insoluble and can be filtered off and refined to pure bone gelatin¹⁵. Procedures developed for use in radiocarbon dating with kilogram-sized bone samples^{9,16} have been scaled down for isotopic studies of the geochemistry of bone using samples of ≤ 5 g.

Carbon isotope analyses were performed on CO_2 directly evolved from hydroxyapatite, and also on CO_2 prepared by a microcombustion procedure from purified bone gelatin—these

Table 1 Carbon isotopic compositions of bone fractions

Sample no.	Species	Location	$\delta^{13}\text{C}$ (‰) Gelatin	$\delta^{13}\text{C}$ (‰) Apatite	Indicated % of C_4 plants in diet
Modern bone					
1	Masai giraffe	Kenya	-21.1	-13.7	0
2	Cape buffalo	Kenya	-10.3	-2.1	75
3	Domestic cow	Kenya	-13.5	-5.5	55
4	Hartebeest	Kenya	-7.8	+0.6	90
5	Grant's gazelle	Kenya	-18.7	-11.7	15
6	Waterbuck	Kenya	-10.0	-2.3	75
7	Zebra	Kenya	-10.5	-0.8	80
8	Thompson's gazelle	Kenya	-13.6	-4.8	55
9	Hippopotamus	Kenya	-11.2	-3.7	65
10	Domestic cow	Kenya	-9.3	-0.5	85
11	Warthog	Kenya	-10.6	-2.7	70
12	Domestic cow	Idaho	-19.6	-10.9	15
13	Domestic cow	Idaho	-22.6	-16.4	0
14	White-tailed deer	Connecticut	-18.9	-12.1	15
$n = 14$ $r = 0.991$ $m = 1.113$			Apatite - Gelatin = $7.94\% \pm 0.93$		
Archaeological bone (<2,000 years old)					
15	Musk ox	N. Canada	-22.1	-14.6	0
16	Arctic hare	N. Canada	-21.2	-13.8	0
17	Bison	New Mexico	-8.7	-0.4	85
18	Bison	New Mexico	-9.4	-0.2	85
19	Unknown animal	Kenya	-6.8	+0.9	95
$n = 5$ $r = 0.997$ $m = 1.061$			Apatite - Gelatin = $8.02\% \pm 0.75$		
Archaeological bone (older than 2,000 years)					
20	Domestic cow	Kenya	-9.7	-0.3	85
21	Unknown animal	Kenya	-6.3	+0.8	95
22	Human	New Hampshire	-17.0	-9.2	30
23	Human	Peru	-19.4	-11.2	15
24	Domestic cow	Kenya	-8.8	-0.6	85
$n = 5$ $r = 0.989$ $m = 0.988$			Apatite - Gelatin = $8.14\% \pm 0.84$		
Fossil bone (older than 10,000 years)					
25	Mastodon	New Jersey	-20.2	-11.7	10
26	Fossil camelid	Idaho	-16.1	-9.1	30
$n = 2$			Apatite - Gelatin = $7.75\% \pm 1.06$		
All analyses					
$n = 26$ $r = 0.991$ $m = 1.067$			Apatite - Gelatin = $7.98\% \pm 0.84$		

**Fig. 1** Relationship between $\delta^{13}\text{C}$ of carbon in bone gelatin and hydroxyapatite. ●, Modern animals; ○, archaeological or fossil animals.

were done using either Micromass 602D or 903 mass spectrometers (at the Geochron Laboratories). Results are reported in standard δ notation relative to the PDB standard¹⁷.

To examine the relationship between $\delta^{13}\text{C}$ of bone gelatin and of hydroxyapatite, bone samples were chosen to represent a wide variety of conditions which might affect the isotopic relationships. Several samples were from animal species which are known to have preferences for either C_3 or C_4 plants^{18,19}, including species that have dietary inputs of almost pure C_3 or C_4 . Samples of differing age were analysed to evaluate the isotopic relationship between gelatin and hydroxyapatite as a function of time and to determine what other geochemical interferences, if any, might cause problems. Samples were selected to provide a broad geographical base for the data, including samples from Africa, various sites in North America, and one site in South America. As far as possible, samples from environments with unusual preservation conditions, ranging from the Canadian Arctic to Kenya, New Mexico and the Peruvian desert, were selected. Among the fossil bone samples are several which have very different conditions of burial and preservation.

The analyses of 26 bone samples are given in Table 1 and the data points are shown in Fig. 1 as a plot of $\delta^{13}\text{C}$ of carbon in hydroxyapatite against $\delta^{13}\text{C}$ of carbon in gelatin. The analyses in Table 1 are derived from animals having almost the full range of plant diets, from 100% C_3 to 100% C_4 dietary inputs. Vogel *et al.*²⁰ have estimated the average isotopic compositions of the basal photosynthetic food webs and assigned $\delta^{13}\text{C}$ values of -26.5‰ and -12.5‰ for C_3 and C_4 plants, respectively. These values represent the total carbon in the plants and may not

accurately represent the carbon that is actually used for metabolism or synthesis of animal tissues. We agree with Tieszen²¹ that there is a need for compound specificity when discussing the minute details of isotopic fractionation. Van der Merwe and Vogel⁶ have determined a $\delta^{13}\text{C}$ of -21.4% for collagen from North American humans with known C_3 diets, and Vogel² reports a value of -21.2% for collagen from African ungulates with pure C_3 diets. Our data for those animals having almost pure C_3 diets indicate a $\delta^{13}\text{C}$ of $\sim -21.5\%$, which confirms the reported shift of $\sim +5\%$ from the basal food web.

Our analyses of gelatin from animals having essentially pure C_4 diets indicate a $\delta^{13}\text{C}$ of $\sim -6.5\%$, which is identical to the value given by Vogel² and close to the value of -7.5% calculated by Van der Merwe and Vogel⁶. The analyses of C_4 -derived gelatin suggest an isotope enrichment of $\sim +6\%$ relative to a C_4 food source. The difference between the $\delta^{13}\text{C}$ of C_3 -derived gelatin and that of C_4 -derived gelatin is $\sim 15\%$ and closely approximates the isotopic difference in the original food materials.

Isotopic analyses of the hydroxyapatite phase of bone are quite different from those of gelatin for the same samples. In all samples the carbon in hydroxyapatite is $\sim 8\%$ heavier than that in the gelatin. Bone apatite derived from pure C_3 diets has a $\delta^{13}\text{C}$ of $\sim -13.5\%$ while that derived from pure C_4 diets has a value of $\sim +1\%$. The hydroxyapatite data thus show the same spread of isotopic values as the gelatin data, but exhibit consistently greater ^{13}C enrichment relative to diet. While protein synthesis to produce collagen apparently induces an isotopic enrichment of $\sim +5\%$ from the basal food web, the carbon incorporated into hydroxyapatite is consistently enriched by $\sim +13\%$ relative to the food web. It seems most likely that the carbon contained in hydroxyapatite is derived from dissolved bicarbonate in blood plasma. Analogous isotopic enrichments exist between marine shells and seawater bicarbonate.

Despite the greater isotopic fractionation between diet and hydroxyapatite, it is clear from Fig. 1 that dietary information is accurately reflected. The relationship between $\delta^{13}\text{C}$ of bone gelatin and $\delta^{13}\text{C}$ of hydroxyapatite is linear and is approximately represented by the equation $\delta^{13}\text{C}_{(\text{apatite})} = \delta^{13}\text{C}_{(\text{gelatin})} + 8$. Figure 1 defines a straight line for the data with a slope very close to unity (correlation coefficient = 0.99). This relationship can be used to estimate the percentages of C_4 plants in the diet using bone gelatin data, bone apatite data, or both together. The particular value of this relationship derives from the fact that it can be used to gain dietary information, using only hydroxyapatite analyses, from older bone samples which no longer contain intact collagen. In this case $\delta^{13}\text{C}_{(\text{apatite})} = \delta^{13}\text{C}_{(\text{food web})} + 13$, and the proportion of C_4 plants in the diet can be obtained directly from the hydroxyapatite axis of Fig. 1.

This model of dietary analysis using bone will have important applications in archaeology, ecology, climatology and other areas and will allow the extension of such studies well back into the Pleistocene.

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Mycorrhizal infection and resistance to heavy metal toxicity in *Calluna vulgaris*

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Although studies of the biology of mycorrhizas have repeatedly demonstrated their capacity to enhance nutrient capture in circumstances where essential elements are present in growth limiting quantities^{1–3}, knowledge of their function in environments containing potentially toxic concentrations of metallic elements is lacking. It is known that mycorrhizal infection can increase the uptake of copper and zinc from soil solutions containing low concentrations of these metals⁴, but any such increase in circumstances of high metal concentration would clearly be disadvantageous. Because of the large number of situations, both natural and man-made, in which heavy metal toxicity can restrict plant growth, it is of great ecological and applied interest to increase our understanding of those factors which permit growth of plants on heavily contaminated soils. One of the most successful colonists of such soils in northern Europe is the strongly mycorrhizal ericaceous plant *Calluna vulgaris*⁵. *Calluna* has a characteristic 'ericoid' mycorrhizal infection which, in contrast to the situation found in vesicular-arbuscular (VA) mycorrhizas, has been shown primarily to enhance nitrogen rather than phosphorus uptake^{6,7}. We have examined the possibility that mycorrhizal infection may influence the resistance of *Calluna* to high levels of heavy metals. The growth, survival and heavy metal content of two races of *Calluna*, one from a metal-polluted site and one from an unpolluted natural heathland, have been compared when plants were grown in the mycorrhizal (M) and non-mycorrhizal (NM) condition in sand cultures supplemented with different levels of copper and zinc. We report here that whereas NM plants show no tolerance of these metals at high concentrations, mycorrhizal infection provides a major degree of resistance to the toxicity and that infection leads to significant reduction of the heavy metal content of the shoot.

Calluna seeds were collected from plants growing on heavy metal-contaminated spoil at Parys Mountain, Anglesey, North Wales, and from natural heathland at Stanton Moor, Derbyshire, England. Seedlings of both races were grown aseptically on water agar and then transferred to sterile soil in Petri dishes. Half of the seedlings of each race were inoculated with an isolate of the mycorrhizal fungus *Peizizella ericae*, which had been obtained from seedlings growing in the original locality of that race. After incubation for 6 weeks in the presence of the appropriate endophyte, M seedlings of both races showed typical heavy endomycorrhizal infection of the ericoid type⁷. At this stage M and NM plants were transferred to pots of sterile acid-washed sand in which they were supplied with a dilute nutrient solution (1/10th Rorison's)⁸ containing either copper or zinc, which are the major metallic pollutants at Parys and at several other sites colonized by *Calluna* in the UK.

The nutrient concentration was selected after preliminary experiments had shown that, with twice weekly addition, macronutrient levels were adequate to sustain growth of both M and NM plants of *Calluna*, which is an intrinsically slow-growing species. P levels were not, however, high enough to cause inhibition of mycorrhizal infection, or precipitation of added metals. The recoverability of N, P and metals was shown in parallel experiments without plants to be greater than 95% at all treatment levels. In addition, levels of P in this medium were the same as those previously reported as 'extractable' from Parys mine spoil and heathland soil⁵, and were thus considered to be both ecologically and physiologically appropriate for this type of experiment. Copper treatments were at 0, 10, 25, 50 and

Table 1 Mean yield (mg) and standard error of plants of two races of *Calluna vulgaris* grown in the mycorrhizal (M) and non-mycorrhizal (NM) condition with different treatments of copper or zinc

Treatment Cu (mg l ⁻¹)	Parys		Stanton		Treatment Zn (mg l ⁻¹)	Parys		Stanton	
	M	NM	M	NM		M	NM	M	NM
0	47.9±2.1	32.0±1.4	48.4±3.0	32.7±2.0	0	47.2±2.4	26.7±3.4	51.9±3.4	24.2±1.6
10	17.6±1.3	1.5±0.4	15.5±2.6	1.3±0.1	25	47.3±4.0*	18.9±2.2†	16.6±2.6	1.7±0.7
25	10.0±0.9	0.9±0.1	13.9±1.1	1.0±0.1	50	23.9±2.6*	1.0±0.1	14.3±1.9	1.4±0.3
50	8.6±0.9	0.6±0.1	8.9±0.8	1.5±0.1	100	15.7±1.2*	0.8±0.2	9.9±0.8	1.4±0.1
75	9.7±0.8	0.7±0.2	6.1±0.7	0.8±0.2	150	15.0±2.0*	0.8±0.1	6.1±0.1	1.5±0.1

All M values are significantly different from NM at $P = < 0.05$.

* Yield of Parys M plants significantly greater than those of the Stanton race at $P < 0.05$; † Parys NM value significantly different from Stanton NM at $P < 0.05$, $n = 6$.

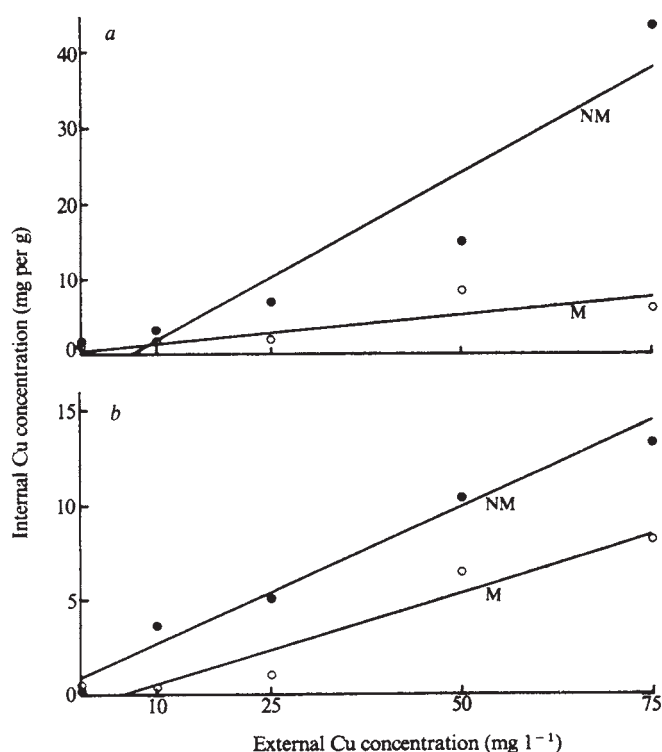


Fig. 1 Copper contents of mycorrhizal (O) and non-mycorrhizal (●) plants of Parys (a) and Stanton (b) races of *Calluna vulgaris* together with fitted regression lines showing the overall relationship between the external and shoot concentrations of the metal. In both cases the slopes of the M and NM regression lines differ significantly at $P < 0.05$.

75 mg l⁻¹ and zinc at 0, 25, 50, 100 and 150 mg l⁻¹. After 12 weeks all seedlings were harvested, their roots and shoots were separated, oven dried, weighed and further processed for analysis of Cu and Zn concentrations by atomic absorption spectrophotometry. The results of the shoot Cu and Zn measurements were subjected to a first order polynomial regression analysis to determine whether external metal concentration influenced tissue concentrations and to compare the magnitudes of any such influence in M and NM plants.

The major response of both races of *Calluna* to addition of heavy metals was that, with one exception (*Calluna* Parys, 25 mg l⁻¹ Zn), the NM plants failed to show any growth in the presence of the metals. Growth of both M and NM control plants was vigorous compared with that of NM plants exposed to metals, which indicates that major nutrient deficiencies were not experienced by the plants. M plants, in contrast, showed some growth even at the highest concentrations of metal and in all cases their yields were significantly greater than those of their NM counterparts (Table 1). The main difference between NM plants of the two races was that mortality was somewhat higher in the Stanton plants. Yield differences between M plants of the two races were small and insignificant in the copper treatments, but yields were significantly higher in Parys M plants in the zinc

treatments. This is the most distinctive difference between the races in the M condition. Because neither metals nor nutrients were precipitated at the concentrations used, it is likely that the main treatment effect in the NM plants is directly attributable to the added metal, rather than to interaction between metals and nutrient availability.

Regression analyses applied to the results of shoot copper (Fig. 1a, b) and zinc (Fig. 2a, b) determinations show that NM plants contain significantly higher contents of these metals in all cases except Stanton zinc. Premature death of Stanton NM plants in the Zn treatment probably contributed to the lower accumulation of Zn at the high treatment levels.

At the highest metal concentrations the root systems of NM plants of both races were so poorly developed that they were not suitable for metal analysis. Metal levels in roots of M plants were analysed and were found to be significantly higher than those of the shoots. This suggests that metals are being retained by the mycorrhizal fungus in the roots.

The severity of the growth inhibition in metal-treated NM plants compared with controls, and its association with a marked increase of metal concentration suggest that metal accumulation is primarily responsible for damage. High metal contents in roots relative to shoots of mycorrhizal plants also suggest that the capacity to maintain growth is associated with metal exclusion and hence avoidance of toxicity. It has been shown in

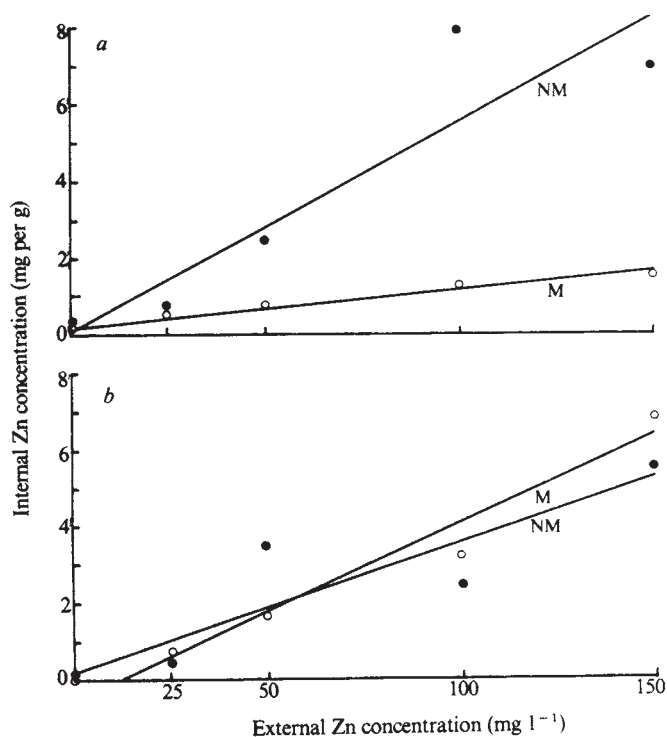


Fig. 2 Zinc contents of M and NM plants of Parys (a) and Stanton (b) races of *Calluna vulgaris*. Other details as in Fig. 1. The slopes of the M and NM regression lines are significantly different in the Parys race ($P < 0.05$) but not in the Stanton race.

the case of copper-tolerant races of the grass *Agrostis tenuis* that the metal is confined to the roots⁹, the main site of the complexing being the cell wall¹⁰. At high levels of metal, however, inhibition of root extension and hence of complexing ability occurs. In these circumstances the presence of an endomycorrhizal fungal symbiont could be of considerable importance. Fungal cell walls, particularly those of the class Ascomycotina to which *Pezizella* belongs, are known to have strong affinities for metallic cations¹¹, and the ericoid infection, which provides a much greater concentration of fungal material in the root than that seen in VA mycorrhizas⁷, would be expected to provide a particularly efficient exclusion mechanism. Elaborate hyphal coils occupy most of the cortical cells of *Calluna* roots in those regions through which absorption occurs and these would provide a greatly increased surface for a retention of metal ions. The capacity, provided by mycorrhizal infection, to avoid metal accumulation may also help to explain the relative success of ericaceous plants on natural heathland soils in which the low pH increases the availability of metallic cations, in particular aluminium and manganese, to levels which are toxic to many non-ericaceous species¹². We are now assessing the ecological and physiological implications of the relationship between mycorrhizal infection and heavy metal resistance in a range of ericaceous species.

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Increased growth of ryegrass exposed to ammonia

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Fluxes of ammonia (NH_3) between the soil/plant system and the atmosphere are components of the nitrogen cycle, but knowledge of them is inadequate^{1,2}. Grassland in the UK annually receives 8.4×10^5 tonnes of fertilizer nitrogen³ and 5×10^5 tonnes of nitrogen through the excreta of grazing ruminants⁴. Emissions of NH_3 from these sources may be 10^5 tonnes annually⁵ and, apart from the loss of a plant nutrient, they may pollute the air. We report here that NH_3 has beneficial effects on growth of perennial ryegrass (*Lolium perenne* L.), apparently through leaf sorption. In air containing NH_3 at $16 \mu\text{g m}^{-3}$, similar to the level above a grazed pasture⁶, sorption would be $48\text{--}224 \text{ ng m}^{-2} \text{ leaf area s}^{-1}$, depending on the density of leaves in the canopy. For grass with a leaf area index of 4, this process would add nitrogen at $6\text{--}28 \text{ g ha}^{-1} \text{ h}^{-1}$. These results support the suggestion⁷ that some of the flux of NH_3 from soil may be sorbed by overlying plants.

The concentration of NH_3 in the air has been found to decrease with increasing height above the soil within the canopy of a pasture and it was suggested that some NH_3 volatilized from a soil may be sorbed by the overlying plants⁷. Recent studies of a

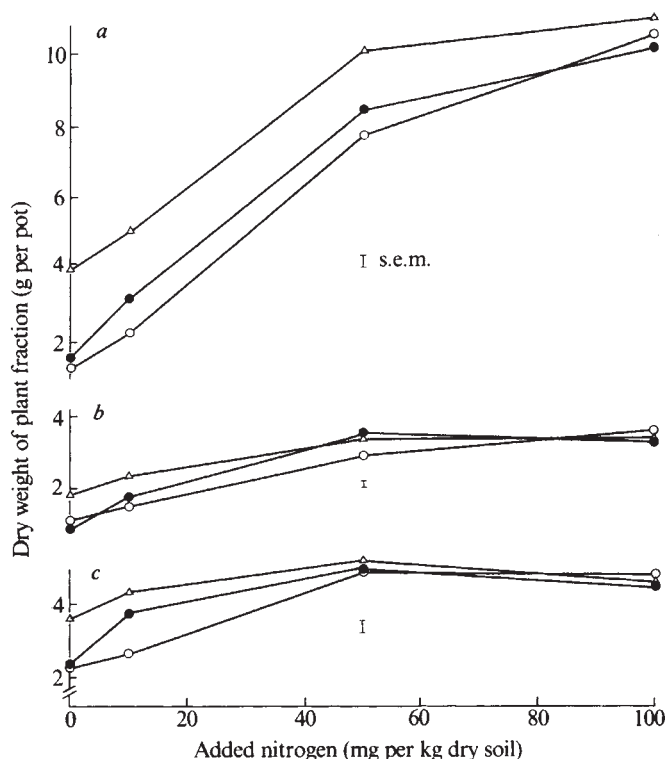


Fig. 1 The dry weight (g per pot) of perennial ryegrass (*Lolium perenne* L.) cv. S23 harvested in three fractions: a, 'shoots', cut 30 mm above the soil; b, 'stubble', bases of shoots cut to soil level; and c, 'roots' (ash-free basis) recovered by sieving and washing. The plants were exposed to filtered air with NH_3 added to give 11 ($\circ$), 148 ($\bullet$), and 550 (Δ) $\mu\text{g m}^{-3}$ throughout a 16-h photoperiod from day 45 after sowing, when they were first cut to 30 mm, until day 71. Fifteen plants were grown in each 15-cm diameter pot containing 4.2 kg of a sandy loam of pH 7.5, described elsewhere¹⁷, to which nitrogen (as NH_4NO_3) was added at either 0, 10, 50 or 100 mg kg^{-1} , together with uniform amounts of phosphorus, potassium, sulphur and magnesium (as $\text{CaH}_4(\text{PO}_4)_2$, KH_2PO_4 , K_2SO_4 and MgCl_2). The soil surface was covered with a 10 mm layer of polyethylene granules to minimize sorption of NH_3 and evaporation of water. Water was added frequently to the pots to bring the soil to an average tension of 7.4 kPa. Sets of four pots, representing the four rates of added nitrogen, were housed in each of nine chambers which provided three replicates of the three NH_3 concentrations. The chambers, which have been fully described elsewhere¹⁸, were modified by the removal of the original perforated ceilings, and they received air at 12 l s^{-1} to give two air changes per min. In these conditions, the boundary layer resistance to the transfer of water vapour measured just within the leaf canopy was 0.04 s mm^{-1} . The concentration of NH_3 was monitored continually in the outlet air and adjusted daily on the basis of determinations both by UV absorption spectrometry (Lear Siegler SM1000 Ambient Air Monitor, Colorado, USA) and by a colorimetric method using Nessler's reagent¹⁹.

number of species^{8–12} have shown that NH_3 may be sorbed by leaves from air containing $4\text{--}14,000 \mu\text{g m}^{-3}$, sometimes with benefit to yield^{8,10}. In some studies^{12,13}, there was no evidence of sorption with NH_3 at $< 4 \mu\text{g m}^{-3}$, rather there was release from senescent leaves.

We examined the effects on plant growth of increments in NH_3 concentration and nitrogen supply to the roots (Fig. 1). The weight of shoots increased with NH_3 concentration, and increased linearly with the addition of nitrogen to the soil, except at the highest rate. Although the stubble and roots (Fig. 1b, c) responded similarly to the shoots, the effects of the treatments were less clear. The concentration of nitrogen in shoots, stubble and roots (Fig. 2a–c) always increased with NH_3 concentration and with the addition of nitrogen to the soil, except at the lowest rate.

When nitrogen supply to the roots was inadequate, the growth of the plants was enhanced by exposure to NH_3 . This

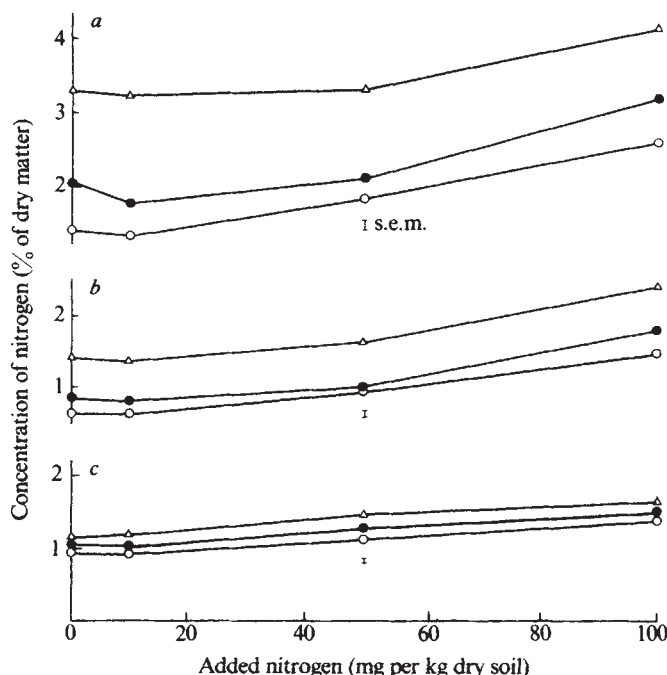


Fig. 2 The concentration of nitrogen (per cent of dry matter) in perennial ryegrass (*Lolium perenne* L.) cv. S23 harvested in three fractions: a, 'shoots'; b, 'stubble'; and c, 'roots', after exposure to filtered air containing NH_3 at 11 ($\circ$), 148 ($\bullet$) and 550 ($\triangle$) $\mu\text{g m}^{-3}$, as described in Fig. 1 legend. The concentration of nitrogen in duplicate oven-dried samples of the three replicate fractions of plant material was determined using an automated Dumas procedure (Coleman 29A Nitrogen Analyser, Illinois, USA).

enhancement was almost certainly through leaf absorption, which has been found for other crops with NH_3 (refs 9, 10) as well as with nitrogen dioxide^{14,15}. The term sorption, as used here, includes absorption into leaf tissue and adsorption on the leaf surface.

The high nitrogen concentration in the shoots exposed to NH_3 at 148 and 550 $\mu\text{g m}^{-3}$ (Fig. 2a) probably results from leaf sorption of NH_3 ; sorption by soil and subsequent root uptake was unlikely to have been important¹⁶. The concentration in the shoots of plants grown without added nitrogen and exposed to NH_3 at 11 $\mu\text{g m}^{-3}$ was 1.4%, it increased to 2.6% with the highest rate of added nitrogen but, surprisingly, increased to 3.3% with exposure to NH_3 at 550 $\mu\text{g m}^{-3}$. This comparison was made between the shoots of plants differing greatly in dry weight. A more appropriate comparison may be made by interpolating the shoot weight of plants grown without added nitrogen and exposed to 550 $\mu\text{g m}^{-3}$ NH_3 (that is, 3.97 g per pot) onto the regression line produced for the shoot weight of plants grown with increased added nitrogen but with NH_3 at 11 $\mu\text{g m}^{-3}$ (Fig. 1a). Thus, a similar shoot weight would have been obtained with nitrogen added at 22 mg kg^{-1} soil; the shoots would have contained 1.5% nitrogen (Fig. 2a), that is, less than half the concentration resulting from exposure to 550 $\mu\text{g m}^{-3}$ NH_3 . This suggests that ammonia-nitrogen sorbed by leaves may be less available for transport and metabolism than nitrogen taken up through the roots. Ammonia may have reacted with the leaf surface, or its accumulation may have had both beneficial and injurious effects on growth.

The rate of sorption of NH_3 , based on: (1) the leaf area, which was assumed, not unreasonably, to have developed linearly, and (2) the extra nitrogen in plants due to NH_3 , may be expressed as a deposition velocity:

$$V_g (\text{m s}^{-1}) = \frac{\text{rate of sorption of } \text{NH}_3 (\mu\text{g m}^{-2} \text{ s}^{-1})}{\text{concentration of } \text{NH}_3 \text{ in air } (\mu\text{g m}^{-3})}$$

where NH_3 concentration is reduced by 11 $\mu\text{g m}^{-3}$, to allow for background in the filtered air. This expression is useful for comparing rates of sorption. Velocity ranged from 3 to 14 mm s^{-1} and was lower for high-yielding plants; a greater

density of leaves presumably increased aerodynamic resistance to NH_3 sorption. In other studies, short-term exposures to NH_3 of isolated plants in chambers have given V_g of 10.4 and 15.4 mm s^{-1} for two grasses¹¹ and 13.0 mm s^{-1} for maize⁹.

The sorption of NH_3 by leaves may be important in reducing potential losses to the atmosphere, especially in heavily fertilized, intensively grazed grassland.

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Sensing of $\Delta\mu\text{H}^+$ in phototaxis of *Halobacterium halobium*

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Phototaxis in *Halobacterium halobium* is governed by two distinct photosystems¹. Cells are attracted by green light, which is absorbed by bacteriorhodopsin, effectively a light-driven proton pump^{2,3}, and repelled by UV and blue light absorbed by a retinal-dependent pigment and by carotenoids^{1,4–10}. One possibility is that the bacterium detects changes in light intensity directly by the interaction of excited absorbent molecules with the tactile system, in which case the phototactic receptors would be analogous to bacterial chemoreceptors¹¹. However, *H. halobium* may sense light changes indirectly as changes in the chemical potential of hydrogen ions ($\Delta\mu\text{H}^+$)^{12,13}; the coincidence of the action spectra for positive phototaxis and photosynthesis^{14,15} indeed support the suggestion that the cells may possess a specific $\Delta\mu\text{H}^+$ receptor, called a 'protometer'. We now describe experiments which indicate that positive phototaxis in *H. halobium* is governed by $\Delta\mu\text{H}^+$ sensing, but that negative phototaxis is mediated by a specific photoreceptor system.

Shortly after an increase in blue light intensity, or a decrease in green light intensity, *H. halobium* cells reverse their direction of travel and, for a certain time period, the rate of reversals remains increased, gradually returning to the basal rate⁸. In peritrichous bacteria (for example, *Escherichia coli*), the adaptation time to a stimulus is directly proportional to the number of excited receptor molecules¹⁶. In the case of *H. halobium* the quantitative significance of the adaptation time is unclear and thus the only adequate method of determining taxis is by recording the fraction of cells that respond to a stimulus. Moreover, it seems reasonable to assume that the first reversal is an immediate response to the chemotactic signal, whereas adaptation reflects the process of inhibiting the chemotactic signal transmission. Therefore the first reversal will provide more accurate information about the reception process than the

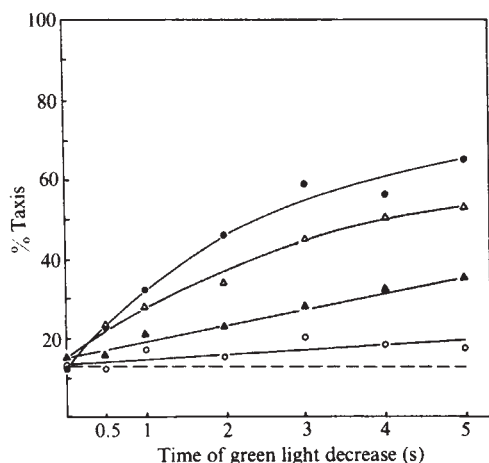


Fig. 1 The effect of inhibiting respiration and ATPase on the sensitivity of *H. halobium* phototaxis. *H. halobium* R₁M₁ lacking gas vacuoles and bacterioruberin (supplied by Dr N. Dencher) were grown in white light at 37 °C on a complex peptone medium (Oxoid) for 96 h. Motility of collected cells was greatly improved by incubation for 24 h in nutrient-free salts media. Cells were observed by phase contrast using a Univar microscope (Reichert). The initial intensity of the green light (500–650 nm) was 9 mW cm⁻². Cell reversal was recorded if the cell reversed within 5 s after insertion of a neutral filter that decreased the intensity by a factor of 1.4. The experimental points are means of 20–60 individual cell recordings. ○, Control; ▲, 0.1 mM DCCD; △, 5 mM KCN; ●, 0.1 mM DCCD and 5 mM KCN. The baseline indicates the level of spontaneous reversals.

adaptation time. If, during phototaxis, bacteria actually sense changes in $\Delta\mu\text{H}^+$ rather than changes in the concentration of excited bacteriorhodopsin, we would expect the inhibitors of oxidative phosphorylation to increase their sensitivity. Indeed, if the respiratory chain is blocked by CN^- and the H^+ -ATPase by DCCD, bacteriorhodopsin remains as the only operating proton pump, and minor changes in light intensity will effect significant changes in $\Delta\mu\text{H}^+$. In the absence of inhibitors the same changes in light, due to the operation of the redox chain, will have little or no effect on the magnitude of $\Delta\mu\text{H}^+$.

There are two ways in which the light stimulus can be varied: by changing the duration of the stimulus, or by altering its magnitude. The first approach was used in the experiment shown in Fig. 1. A given decrease in light was applied to the cell suspension for various periods of time. The sensitivity of phototaxis seemed to be much higher in the presence of CN^- and DCCD than in the absence of inhibitors. Oxygen consumption,

Table 1 Responses of *H. halobium* cells to monochromatic and mixed light

Illumination	Per cent cells reversed in 3 s	Motility rate (μs^{-1})
Orange background: 0.12 mW cm ⁻² , $\lambda < 520\text{ nm}$	<5	1.5
Increase in orange light to 7.2 mW cm ⁻²	<5	2.7
Orange background + blue light ($\lambda < 400\text{ nm}$), 0.03 mW cm ⁻²	73	1.5
Increase in orange light to 7.2 mW cm ⁻² + blue light, 0.03 mW cm ⁻²	42	2.8
Orange background + intense blue ($\lambda < 490\text{ nm}$)	100	2.7
Increase in orange light to 7.2 mW cm ⁻² + intense blue ($\lambda < 490\text{ nm}$)	100	2.7

Cells were incubated in a salt medium with NaCl mainly substituted by KCl²³. The slide was kept at 37 °C using a Reichert Biotherm microscopic thermostat. Blue light with $\lambda < 490\text{ nm}$ (bottom two lines) was saturating for phototaxis.

monitored using a Clark electrode, was completely inhibited by 5 mM CN^- , and light-driven ATP synthesis measured using firefly luciferase was found to be depressed by 90% in cells preincubated for 30 min with 0.1 mM DCCD (data not shown). In control cells lacking inhibitors a significant taxis response was observed only on a strong decrease in light. We found that the smaller the changes in light intensity, the greater the difference in phototaxis sensitivity between cells in the presence and absence of inhibitors, respectively (Fig. 2a). Control cells showed only a slight response to a twofold decrease in light intensity, while in the presence of inhibitors (closed circles) they responded with an 80% taxis effect.

These data indicate that phototaxis sensitivity strongly increases in conditions in which a sudden decrease in illumination produces a significant change in the energy level. However, the overall phototaxis could result from the mutual action of a protometer and a truly specific bacteriorhodopsin light receptor. A given decrease in light intensity would cause a given drop in the $\Delta\mu\text{H}^+$ level in cells with inhibitors. A stronger decrease in illumination would result in the same decrease in $\Delta\mu\text{H}^+$ in the control bacteria. As the decrease in $\Delta\mu\text{H}^+$ is the same in both cases, although the changes in light intensity differ, an equal phototaxis response would rule out a specific photoreceptor mechanism. If the phototaxis activity were higher in the strongly illuminated sample, that is, in that lacking inhibitors, this would suggest a specific light-sensing process.

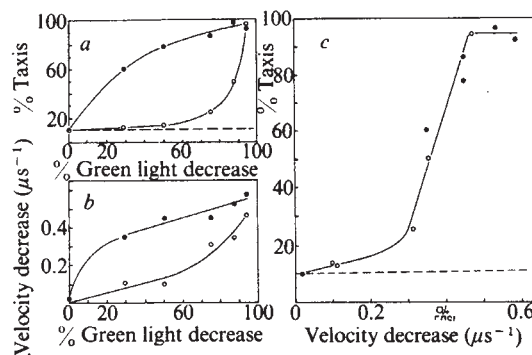


Fig. 2 a, The correlation between taxis activity and changes in motility rate with decreases in illumination. Conditions essentially as described for Fig. 1 legend. Initial intensity of the green light (500–650 nm) was 9 mW cm⁻². Reversals were recorded after the insertion of various neutral filters for 5 s: ○, control; ●, 0.1 mM DCCD + 5 mM KCN. b, Motility rate was recorded by means of an ocular scale and a stopwatch before and after insertion of the same neutral filters as were used in a. The ordinate is the difference between control motility rate (2.4 μs^{-1}) and motility rate after a decrease in light: ○, control; ●, 0.1 mM DCCD + 5 mM KCN. c, Plot combining the data from experiments a and b.

As bacterial motility was found to be directly supported by $\Delta\mu\text{H}^+$ (refs 17–20) and its rate to be positively correlated with the magnitude of $\Delta\mu\text{H}^+$, we used motility rate as a probe for $\Delta\mu\text{H}^+$. Figure 2 shows results of an experiment in which both motility rate and taxis were studied as a function of light intensity. When the phototactic response was plotted against the decrease in swimming velocity (Fig. 2c), the experimental points obtained both with control cells and those with inhibitors all lay on the same curve. This means that differences in light intensity changes *per se* are non-essential and that phototaxis is governed by changes in the energy level, presumably by $\Delta\mu\text{H}^+$.

It seems to be important to correlate phototactic response with the absolute values of changes in $\Delta\mu\text{H}^+$, although it is technically difficult to measure $\Delta\mu\text{H}^+$ both rapidly and quantitatively in *H. halobium* in given conditions. Experiments on this are being done.

One may think that ATP, not $\Delta\mu\text{H}^+$, is the principal effector in phototaxis. The possible existence of a specific ATP receptor in enterobacteria has been discussed elsewhere¹³. However, this is not the case in *H. halobium*, as the addition of DCCD

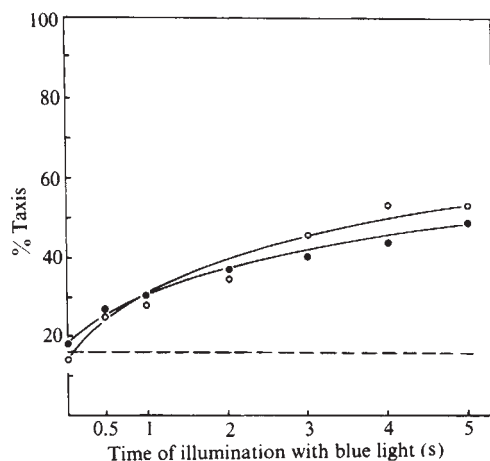


Fig. 3 The effect of inhibiting respiration and ATPase on the avoidance of blue light. Conditions were essentially as described in Fig. 1 legend. The intensity of the green background (500–580 nm) illumination was 36 mW cm^{-2} . Blue light stimuli ($\lambda < 400 \text{ nm}$, $1.4 \times 10^{-2} \text{ mW cm}^{-2}$) were applied through an incident light illuminator of the Univar microscope equipped with a 200-W mercury arc.

increased, rather than inhibited, the sensitivity of the phototaxis system.

The approach used in studying positive phototaxis was applied to test the negative phototaxis system. Blue light is known to affect the bacteriorhodopsin photocycle and thus to decrease $\Delta\mu\text{H}^+$ (ref. 21). Wagner *et al.*²² found that UV light (360 nm) partially inhibited K^+ uptake by *H. halobium* cells. They speculated that the behavioural response to blue light might be due to a decrease in membrane potential²². However, the inhibiting action of blue light, with a maximum at 412 nm, cannot be solely responsible for negative phototaxis, which has two main maxima, at 280 and 370 nm¹. Dencher⁵ and Hildebrand⁶ suggested that the blue light acts by decreasing the membrane potential, opening an ion channel associated with the pigment P_{370} . If the repelling action of blue light were indeed due to a decrease in $\Delta\mu\text{H}^+$, one would expect the sensitivity of phototaxis to depend on the activity of the cellular proton pumps and it should thus be increased by CN^- and DCCD.

Pulses of blue light with durations varying from 0.5 to 5 s were applied to cell suspensions with and without CN^- and DCCD (Fig. 3). Avoidance of blue light did not show a dependence on the ability of the stimulus to change the energy level. To test further the possible dependence of negative phototaxis on changes in $\Delta\mu\text{H}^+$, a simultaneous stimulation by blue and green light was used; effects were shown to sum up algebraically⁸. Cells were partially de-energized by preincubation in dim light. As the K^+ gradient may support the energization²³ and motility²⁴ in *H. halobium* cells for a prolonged period of time, bacteria were incubated in a medium with a high KCl concentration. After incubation for 2 min the motility rate decreased from 2.7 to $1.5 \mu\text{ s}^{-1}$ and could be brought to the initial level by illuminating the suspension with intense light for 10 s (Table 1). A mixture of blue and orange light elicited a reversal of 42% of *H. halobium* cells in 3 s. This reversal was accompanied by a significant increase in the motility rate, indicating that the repellent response of the cells to blue light is not due to a decrease in $\Delta\mu\text{H}^+$. Increase in blue light alone produced 73% reversal of cells, which indicates that the effect of mixed light was a summation of an attractant (orange) and repellent (blue). Blue light alone had no effect on motility rate. The increase in motility rate was the same whether the intensity of orange light was increased alone or in combination with blue. If a high intensity blue light was used, the rate of reversals became independent of a simultaneous increase in orange light. The data shown in Table 1 clearly demonstrate that negative phototaxis does not result from changes in $\Delta\mu\text{H}^+$.

The differences in the mechanisms of positive and negative phototaxis in *H. halobium*, that is, an indirect sensing of green light by means of a protometer and a direct sensing of blue light by a specified photoreceptor, would explain the striking differences in light sensitivity. Blue and UV light evoke a response at 1/10 and 1/500 the intensity of green light¹. This difference seems to be natural if we consider that the more sensitive system operates as a specialized photoreceptor.

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Control of mucus hydration as a Donnan equilibrium process

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The physical properties of the mucus secreted by the uterine cervix of primates and other animals undergo periodic changes which are important in the regulation of fertility. During the luteal phase of the sexual cycle, cervical mucus is thick, rubbery and impenetrable by spermatozoa; by the time of ovulation, it is a watery gel easily penetrated by spermatozoa. Although these changes in the rheological properties of mucus have been attributed to variations in the covalent cross-linking between long chains of glycoproteins, the main macromolecular constituent of cervical mucus^{1,2}, recent studies^{3,4} suggest that the macromolecular matrix of cervical mucus is probably not covalently cross-linked. Rather it is an entangled random network of long mucins³, in which case the observed changes in the physical properties of mucus would be explained by varying degrees of hydration, the mechanism of which is not understood⁵. We now describe experiments which show that the hydration of cervical mucus may be controlled by a Donnan equilibrium process.

Polyionic gels can behave as a semi-permeable membrane and become hydrated following a Donnan equilibrium⁶. As mucins are polyionic species, mucus hydration could also follow a Donnan equilibrium⁷ in which the glycoproteins may function both as structural components of a stable continuous network, analogous to a semi-permeable membrane (which prevents the movement of polyions), and as the entrapped polyionic species that generate the osmotic drive. A typical feature of Donnan equilibria is the dependence of the movement of water on the ionic concentration and pH of the solvent. As the transport of ions in mucus secretory epithelia is known to be physiologically regulated⁵, it seems reasonable to propose that if mucus swelling follows a Donnan equilibrium, mucus hydration could be phy-

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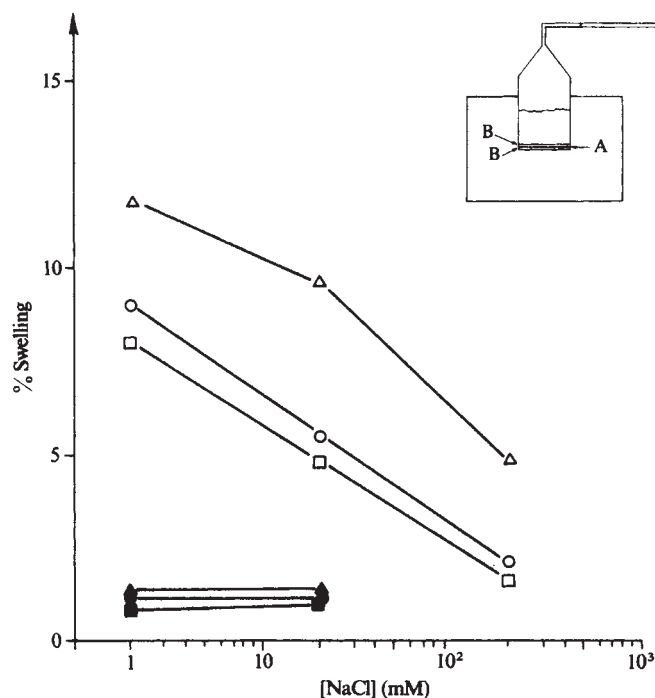


Fig. 1 Relationship between relative (%) swelling of cow cervical mucus at pH 7 and NaCl concentration in the swelling medium for three different samples of luteal (open symbols) and three samples of oestrous mucus (solid symbols). The inset is a diagram of the swelling chamber. Exchange of H_2O , ions and low molecular weight species is established across the Nucleopore filter membrane (A), which is sandwiched between two washers made of stainless steel fine wire mesh (B). See text for more detailed description.

biologically regulated by movement of water and electrolytes (including H^+) across the epithelia.

Six samples each of luteal and oestrous fresh cow cervical mucus were obtained from the Animal Research Facility of Carnation Farms in Washington. The stages of the oestrous cycle were determined by rectal palpation. Mucus swelling was measured as the relative volume expansion of mucus, using an osmometer-like swelling chamber (Fig. 1 inset). In this chamber 0.1 ml of mucus sits on a diaphragm of Nucleopore filter (0.8 μm pore size) at the bottom of a small cylindrical container. The upper portion of the cylindrical container, above the mucus, is filled with silicone oil, forming a single fluid column that ends in a capillary tube (10 cm long and 16.7 μl volume). The water transfer between the mucus and the swelling solution is established across the filter and the volume expansion of the mucus is measured by the displacement of the oil meniscus in the capillary tube. In these experiments, the bottom of the mucus container was immersed in 50 ml of swelling medium which was continuously agitated at room temperature. Swelling medium was a water salt solution containing 2, 20 or 200 mM NaCl at neutral pH, or 150 mM NaCl at pH 8.0, 7.6, 6.99 or 6.46. The pH was adjusted with a phosphate buffer by changing the ratio of monobasic to dibasic sodium phosphate, but keeping the total phosphate concentration constant at 5 mM.

Figure 2b shows the typical time course of swelling of oestrous and luteal mucus in a swelling solution containing 150 mM NaCl at pH 6.46. Note that oestrous mucus demonstrates a very small amount of swelling whereas luteal mucus allows a 13% swelling at equilibrium in ~200 min. Figures 1 and 2 show the relationship between swelling at final equilibrium and salt concentration, and swelling and pH, respectively, in both oestrous and luteal mucus. Whereas oestrous mucus does not undergo swelling, luteal mucus demonstrates significant and irreversible swelling inversely proportional to the log [NaCl] in the swelling medium, and at constant salt concentration (150 mM NaCl), linearly proportional to the pH of the swelling solution.

A unique feature of the Donnan equilibrium is the redistribution of water and ions in the presence of a barrier that limits the mobility of an electrostatically charged (polyionic) molecule. In the most common experimental demonstration of a Donnan equilibrium, this barrier is a semipermeable membrane⁷. However, there are other ways of hindering the mobility of a polyionic molecule. For example, in polyionic gels the mobility of an electrostatically active polymer is limited by topological constraints (cross-links and/or entanglements) between long chains of the polymer itself⁶. In the particular case of the mucus, the topological constraints of the molecular matrix prevent the glycoproteins from diffusing out of the gel. Therefore, in mucus, glycoproteins have a dual role as the structural elements of the entrapping molecular network, and as the entrapped polyions responsible for the osmotic drive.

The dependence of the movement of water on the concentration of ions and the pH of the solvent is a typical characteristic of a Donnan equilibrium and is due to the interaction of polyionic species. The phenomenon can be easily visualized by considering the glycoproteins as electrostatically charged chains. Although the role of ions in water movement depends on their colligative effects, pH affects water movement by changing the total electrostatic charge of the entire chain in a way that is

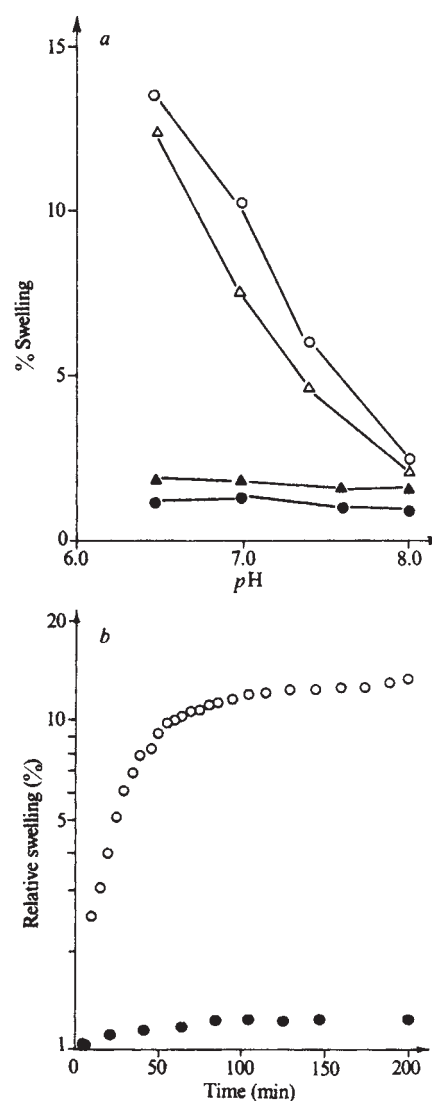


Fig. 2 a, Relationship between relative (%) swelling of cow cervical mucus and pH, in a swelling medium containing 150 mM NaCl. Samples $\circ$, Δ are luteal, and Δ , $\bullet$ are oestrous mucus. b, Time course of swelling as relative (%) volume increase for oestrous ($\bullet$) and luteal ($\circ$) cow cervical mucus in a medium containing NaCl 150 mM, pH 6.46, at 20 °C.

dependent on the ionization constant of dissociable groups in the glycoproteins. The dominant dissociable groups in mucus glycoproteins are axial sialic acidic groups in the oligosaccharide side chains of the molecule⁸. Therefore, the decrease in mucus hydration with increasing pH and/or ionic concentration found in these experiments is indeed predicted by the Donnan equilibrium.

The rheological properties of mucus are important in trans-cervical migration of sperm into the uterus as well as in mucociliary transport in the airways. Hydration of mucus is a critical determinant of these properties⁹. Mucus is discharged from the secretory granules of the cell as densely packed, membrane-free boluses¹⁰ which are then hydrated in some unknown way⁵. The evidence presented here shows that luteal cow cervical mucus can be hydrated according to the Donnan equilibrium. Within this equilibrium, the changes of pH and salt concentration that effectively control mucus swelling *in vitro* are in the same range of physiological values reported in cervical mucus of cows and women. Although the actual fluctuations of NaCl in cervical mucus remain a controversial issue^{11,12}, there are indications that the onset of mucus hydration (and ferning formation) is preceded by a decrease in pH which also coincides with the earliest increases in urinary oestrogens^{13,14}.

These findings, together with our previous observations³, point to a new concept in understanding the physiological control of mucus hydration and rheological properties. They predict that mucus boluses consisting of densely packed entanglements of mucins could be swollen in the extracellular medium following a Donnan equilibrium process. Accordingly, the movement of ions (including H⁺), soluble proteins and water across the mucosa, as well as the amount of soluble proteins and ions within the secreted mucus, could be the variables under physiological control which would determine mucus hydration, and thereby its rheological properties. It has been shown that secretions of soluble proteins in the cervix¹⁵ and uterus are responsive to hormonal control (M. K. Postle and R. B. Heap, personal communication). However, the role of the soluble proteins found in the mucus has not been clearly established^{15,16}. The model we propose suggests an osmotic function for the soluble proteins as potentially entrapped polyions and is consistent with the available evidence, that 'unswollen' luteal mucus contains high amounts of soluble proteins, while hydrated oestrous mucus contains small amounts¹⁵. In the latter case, the mucus molecular network would be expanded allowing soluble proteins to undergo further dilution and escape the gel matrix.

Although there is no direct evidence to validate the predicted hormonal or neurohormonal control of the movement of water, H⁺ and electrolytes across the cervical mucosa, it has been shown that in the respiratory mucosa the movement of water, Na⁺ and Cl⁻ is responsive to hormonal or neurohormonal stimulation⁵.

The present experiments were limited to an exploration of the interaction of cow cervical mucus with NaCl at different pH. However, with only minor constraints, the concept that mucus hydration could be regulated by the Donnan equilibrium remains valid for other types of mucus and other ionic influences. For instance, an interesting implication of this concept is the potential existence of a Donnan-equilibrium-coupled system to delay enzymatic catalysis. Indeed, the application of synthetic polymer gels to entrap enzymes (including proteases) is well known in engineering¹⁷ and is beginning to find wide industrial application.

Another significant prediction of the proposed model regards the pathophysiology of cystic fibrosis, where mucus would be permanently unswollen probably due to deficiencies in the control of the swelling medium and/or the polyionic composition of the secreted mucus. Although there is no direct evidence for defective trans-epithelial movement of water and ions in cystic fibrosis, it has been shown that NaCl is markedly increased in exocrine sweat, and that calcium transport seems to be decreased in red cells and fibroblasts and increased in exocrine secretions in these patients¹⁸⁻²⁰.

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The fate of inner cell mass and trophectoderm nuclei transplanted to fertilized mouse eggs

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When nuclei from 12-18-cell mouse embryos have been injected into fertilized mouse eggs^{1,2}, donor and host chromosomes in those eggs that survived the operation combined to form a single tetraploid nucleus. Some of the resulting embryos developed into morphologically normal blastocysts. At the 8-cell stage, mouse blastomeres are still totipotent³ and are indistinguishable from one another. By the blastocyst stage, inner cell mass and trophectoderm have separated into distinct cell lineages that differ in morphology, physiology⁴ and biochemistry⁵. The fetus develops entirely from inner cell mass while the trophectoderm contributes only to placenta and fetal membranes; it is unclear when this is determined. Aggregation experiments suggest that some blastomeres are still labile at the late morula/early blastocyst stage^{6,7}. If this was the case at least some of the donor cells used in my previous nuclear transfers might have been still totipotent. However I report here that nuclei from trophoblast and inner cell mass are different from one another, as judged by the developmental fate of fertilized mouse eggs to which they are transplanted.

Fertilized one-cell eggs were obtained from spontaneously ovulating CR and Q females mated with syngeneic males. Eggs were collected between 10.00 and 12.00 h on the day of mating. Donor cells were from 3.5-4-day old CR, Q and MF1 blastocysts from which the zona pellucida had been removed by pronase. The mural trophectoderm was dissected from the blastocysts microscurgically⁸; inner cell mass tissue was obtained by either microsurgery or immunosurgery⁹. The severed mural trophectoderm and inner cell mass tissues were placed for ~30-40 min in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (a 0.02% solution of EDTA was occasionally also used) and pipetted using a narrow pipette. Single cells taken from the

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trophectoderm or inner cell mass were drawn into a fine glass micropipette to disrupt the cell membrane; the nucleus and the associated cytoplasm were then injected into fertilized eggs at the early pronuclear stage^{1,2}. Before and during the operation the host eggs were kept in medium 16 (ref. 10) containing 10 g ml^{-1} cytochalasin B to facilitate microsurgery^{11,12}. The eggs which survived the operation were cultured in medium 16 for 48–90 h and most of the resulting embryos were examined in air-dried preparations¹³.

Trophectoderm and inner cell mass nuclei looked intact and were similar in appearance at the time of injection, and therefore there was no reason to believe that trophectoderm nuclei suffered more damage than inner cell mass nuclei during recovery; the proportion of eggs degenerating immediately after injection was similar after transplanting either type of nucleus. However, Table 1 shows that the fate of the two classes of eggs was different. Eggs that had received inner cell mass nuclei developed similarly to those injected with morula cell nuclei². Of the 16 that were cleaved, eight developed to the late morula and blastocyst stage, and appeared normal. In six embryos (one blastocyst, two morulae, one 4-cell and two 6-cell embryos), tetraploid metaphase plates were found. This shows that nuclei originating from these two types of cells (that is, morula and inner cell mass nuclei) can contribute their chromosomes to those of an egg, thus both sets can form a common metaphase plate. The embryo carrying such a tetraploid hybrid nucleus can develop at least to the blastocyst stage. Thus, although the degree of activity of the transferred genome is unknown, one may suppose that the nuclei of such cells do not change irreversibly (if they do so at all) during preimplantation development and their redifferentiation is possible in the egg cytoplasm. In contrast, when the donor nuclei were trophectodermal in origin, donor and host chromosomes still integrated into a single nucleus but development was arrested after two to three cleavages, and no late morulae or blastocysts were formed.

The failure of trophectodermal nuclei to support normal development agrees with the recent findings of Illmensee and Hoppe¹⁴. Mouse eggs enucleated after fertilization and injected with inner cell mass nuclei developed not only into normal blastocysts but, after transfer to pseudo-pregnant foster mothers, into normal fertile mice. In contrast, eggs which received trophectoderm nuclei never developed beyond the first few cleavage divisions.

One of the characteristics of mural trophectoderm (that is, that part of trophectoderm not overlying the inner cell mass) is that it undergoes little or no further cell divisions after the blastocyst stage⁸. DNA accumulation continues by a process of endoreduplication¹⁵, resulting in trophoblast giant cells with large nuclei that may contain several hundred times the haploid DNA content. To determine whether endoreduplication occurred after injection of trophectoderm nuclei into fertilized eggs, the embryos arrested in cleavage (see Table 1) were fixed, air-dried and stained with Feulgen, together with a control series of embryos recovered from the uterus on the fourth day of gestation; DNA determinations were then carried out on the nuclei of experimental and control embryos by microdensitometry. There was no evidence of endoreduplication

Table 2 DNA content of the nuclei in mouse embryos arrested in cleavage after injection of a trophectoderm nucleus into the fertilized egg

Group	No. of embryos	Mean cell number	No of nuclei with DNA content of				Total
			2C	4C	4–8C	8C	
Trophectoderm nuclear transfer	4	6.5	1	10	6	9	26
Control	7	32.7	60	62	0	2	124

Embryos were fixed and air-dried; a cut surface of the mouse liver was pressed on to the end of the slide, and then Feulgen-stained. DNA determinations were made using an M85 Vickers integrating microdensitometer. Liver nuclei gave discrete peaks for 2C, 4C, 8C, 16C, and 32C DNA values, where C is the haploid DNA content; embryo nuclei were assessed against these standards. In the experimental series, each nucleus was measured three to four times; the readings obtained were very consistent. In the control series, where cell number was greater, some nuclei overlapped and were therefore not measured.

(Table 2): the DNA contents of the nuclei were in the 4–8 C (where C is the haploid DNA content) range expected in tetraploid cells arrested in the G₁ or G₂ stage of the cell cycle, respectively, while the diploid controls were almost all in the 2–4 C range.

Although the transplanted trophectoderm nuclei did not undergo endoreduplication as they would during normal development, the observation that cleavage in this series was arrested after two or three cell divisions suggests that these nuclei not only have a restricted potential for further division themselves, but that, following their own programme, they may impose it on the host cell and block its divisions as well. Alternatively, some factor in the trophectoderm cytoplasm, which in this technique is injected into the fertilized egg along with the donor nucleus, may inhibit further development. Also, the lack of endoreduplication in such embryos might be the result of the influence of egg cytoplasm. Thus it is likely that the behaviour of trophectoderm transplants is the result of complicated interactions between host and donor nuclei and cytoplasm.

In Amphibia, nuclei even from differentiated cells have, after transplantation to enucleated eggs, been able to support development up to the late tadpole stage, which consists of a wide variety of differentiated tissues¹⁶. The present observation does not necessarily indicate any difference between Amphibia and mammals. Trophectoderm is a highly specialized tissue, with a limited lifespan¹⁷ and no share in forming the embryo itself. It may be that nuclei from tissues differentiated within the embryo will prove better able to participate in normal development.

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Table 1 *In vitro* development of fertilized mouse eggs injected with inner cell mass or trophectoderm nuclei

Origin of donor cells	No. of eggs injected	No. of eggs surviving (%)	Stage after 48–90 h in culture				M	B
			1 cell	2–4 cell	5–8 cell			
Inner cell mass	142	21 (14.7)	5	3	5	5	3	
Trophectoderm	167	18 (10.7)	5	7	6	0	0	
12–18-cell embryos, data from Modlinski ²	166	15 (9.0)	5	2	0	3	5	

M, morulae; B, blastocysts.

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Dendritic territories of cat retinal ganglion cells

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Ganglion cells have to cover the retina with their dendritic fields so that every point of the visual space is 'seen' by at least one ganglion cell of each physiological type. Using neurofibrillar methods it has now become possible to stain all the α -ganglion cells so that their dendritic network can be analysed¹. Both subpopulations of α -cells, corresponding physiologically to ON-brisk-transient cells²⁻⁵ and OFF-brisk-transient cells, achieve a uniform and independent coverage of the retina. The cell bodies are arrayed in a regular mosaic^{1,6} and the dendritic fields adapt to the available space. It is suggested that during development interaction between neighbouring ganglion cells of the same functional type regulates their dendritic field sizes.

There are more or less economical ways in which nerve cells can overlay a planar surface with their dendrites, given the requirement that every point should be covered by at least one dendritic field. For cells arrayed in a hexagonal or square lattice with spacing ' a ', circular dendritic fields of radius $a/\sqrt{3}$ or $a/\sqrt{2}$ would provide at least onefold coverage (Fig. 1). Were the cells distributed at random, onefold coverage could be achieved either by rather large dendritic fields of uniform size or by interaction between neighbouring cells, where adjustment of the dendritic field size to the local intercellular separation would give rise to a broad and irregular range of dendritic fields.

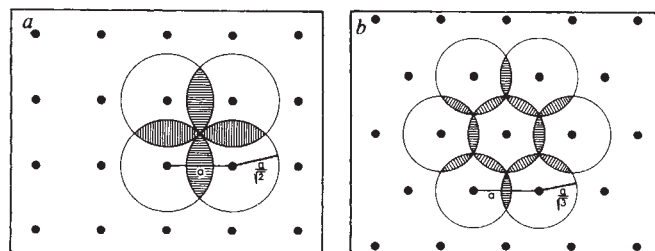


Fig. 1 *a*, Square lattice of cell bodies. The smallest circular dendritic fields, which would provide a onefold coverage, have a radius of $a/\sqrt{2}$. In the hatched area the dendrites of two cells overlap. *b*, Hexagonal array of cell bodies. In this instance at least onefold coverage is ensured by dendritic fields of radius $a/\sqrt{3}$.

Ganglion cells of the retina are a good model system for study of the organization of such a sheet of neurones because their cell bodies and their dendritic fields approximate to a single plane. In the cat retina several different morphological⁷ and physiological classes⁸ of ganglion cells have been defined. With neurofibrillar staining methods all α -ganglion cells were stained to their whole extent, that is, cell body and dendritic tree¹. It was shown that for all OFF-centre cells the dendritic branches lie close to the inner nuclear layer, whereas for ON-centre cells the branching plane is $\sim 10 \mu\text{m}$ closer to the ganglion cell layer^{1,9}. This then constitutes a functionally homogeneous population of neurones which is accessible to quantitative investigation.

Figure 2*a* isolates the ON-population of α -cells and shows the field homogeneously covered by the dendrites. That the cell bodies are distributed nonrandomly was shown by analysis of their nearest-neighbour distribution, which is gaussian^{1,6}. The degree of dendritic overlap is rather small; an area centred on

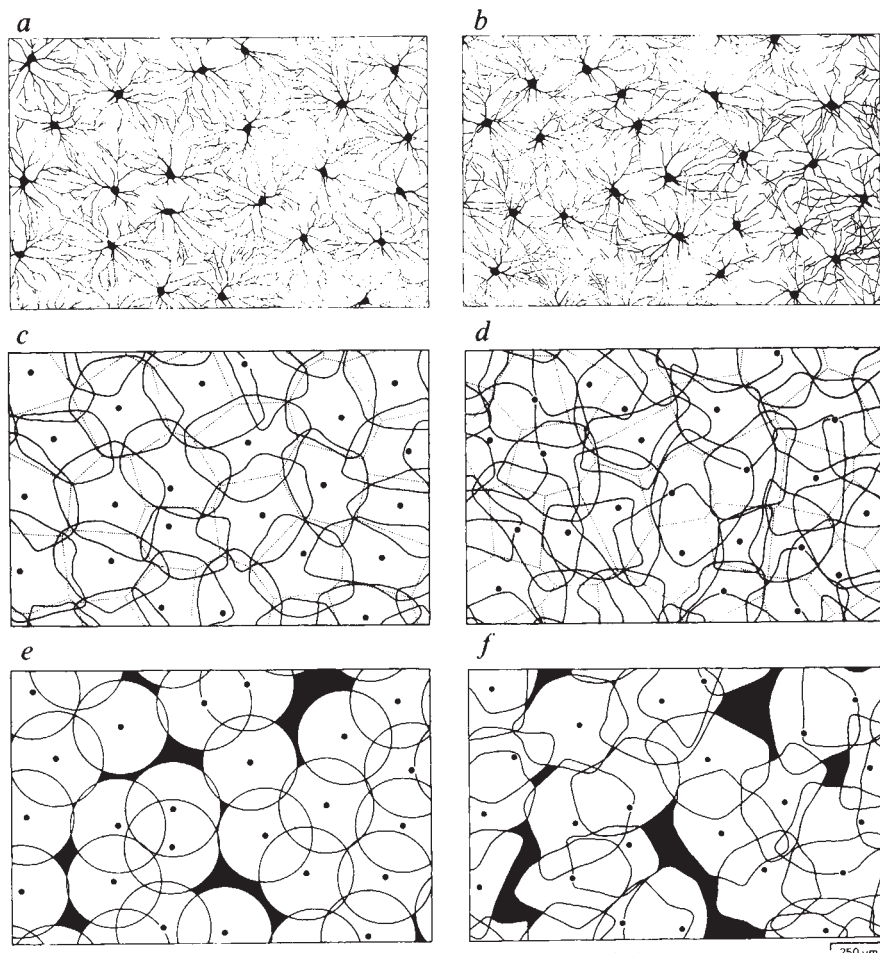


Fig. 2 *a*, The population of ON- α -ganglion cells taken from an area $1.7 \times 1.2 \text{ mm}$ of a Bodian-stained cat retinal whole mount (eccentricity 4 mm). *b*, Same field as in *a*, but only the OFF- α -ganglion cells are shown. *c*, Solid curves are the contours of the dendritic fields of the ON- α -cells shown in *a* and the dots indicate the cell body locations. The dotted polygons are Dirichlet domains, which were constructed for each cell body as described in the text. *d*, Same diagram as in *c* but for the OFF- α -cells. *e*, Coverage of the retina with hypothetical circular dendritic fields based on the mosaic of ON- α -cells; the black areas are not covered. *f*, Every ON- α -cell dendritic field is substituted by its right-left mirror image with the cell body unchanged.

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the cell body of each neurone is not covered by the dendrites of neighbours. OFF-cells show the same arrangement (Fig. 2b). Dendritic fields were defined by connecting the outermost dendritic tips of every cell by a smooth closed curve (Fig. 2c, d). The dendritic fields are irregular. In some cases there is an apparent correlation between the dendritic tree of a cell and the space not occupied by neighbours.

The concept of Dirichlet domains¹⁰, which cover the area by contiguous convex polygons, is introduced in Fig. 2c and d for comparison. Around every cell body a circle of 500 μm diameter was constructed and the intersecting lines of neighbouring circles were drawn. Dirichlet domains subdivide the area into small territories, one for each cell, with the property that every point in a given territory is closer to its own cell body than to any other. Thus, a dendritic growth model in which the dendrites grow out of the cell bodies at the same time and rate and stop growing when they touch dendrites of neighbouring cells can be described in terms of Dirichlet domains, although they do not account for overlapping dendritic trees. The Dirichlet domains are a rather good approximation to the dendritic fields. Basic features, such as the orientation of the long axis, nearly always agree as between the dendritic field and its Dirichlet domain. This implies that there might be some mechanism of mutual regulation of the dendritic growth of neighbouring ganglion cells of the same type.

Another test for the correlation between the dendritic field dimensions and the available space is shown in Fig. 2e. Circular fields of the average dendritic field diameter (399 μm) were constructed around each ON-cell perikaryon. Such uniform domains leave gaps and the coverage is consequently incomplete and not very homogeneous (Fig. 2e). When, with the cell body in its original position, every dendritic field is replaced by its mirror image, the coverage is again very inhomogeneous, uncovered gaps are found next to areas where the dendritic fields of several ganglion cells overlap (Fig. 2f).

We infer from these observations that some kind of local regulation which defines the shape and size of the dendritic fields must prevail during development. This process must be highly specific because ON-centre cells influence neighbouring ON-centre cells but not OFF-centre cells and vice versa. The same mechanism could regulate the increase of the average dendritic field size with distance from the central area and so balance the decreasing α -cell density. A similarly specific process must be active at the cell body level to regulate nearest-neighbour distances¹.

Every point in the field represented by Fig. 2c is covered by an average of 1.4 ON- α -cells (average dendritic field area $\times$ density). For cells arrayed in a square lattice with circular dendritic fields, the geometrical constraints shown in Fig. 1a lead to an average coverage of 1.57 to ensure at least a onefold coverage everywhere. Arrangement of the ganglion cells in a hexagonal lattice would require an average coverage of 1.21. This shows that although the ganglion cells are in fact distributed less precisely than in a perfect lattice, their dendritic trees cover the given field of Fig. 2a more economically than in a square array and close to the theoretical limit possible in a hexagonal lattice.

The size and shape of an α -cell's receptive field centre, although slightly larger, closely corresponds to its dendritic field³. Therefore, these morphological results imply a complete and economical coverage of either functional subclass. Two-dimensional sheets of neurones are not only found in the retina; arrangement in layers is common in many parts of the central nervous system. The multilamellar structure of the cerebral cortex is an example and it would be interesting to see whether identical functional units there exhibit the same kinds of territorial attributes and regular intercell spacings.

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Androgen increases formation of behaviourally effective oestrogen in dove brain

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Oestradiol-17 β (E_2) formed from testosterone in the brain is thought to be involved in the hormonal control of male sexual behaviour in some mammal species^{1–3}. In the male dove, *Streptopelia risoria*, the aromatase system in the preoptic area (POA) is very active in converting testosterone to E_2 (ref. 4). This oestrogenic metabolite has specific effects on male nest-orientated courtship patterns, which are mediated by an oestrogen-sensitive system in the preoptic-anterior hypothalamic area of the brain⁵. The preoptic aromatase system is likely to be important in the regulation of androgen action on male courtship behaviour, as no circulating oestrogen can be detected in the blood plasma of the male⁶. We report here that the level of aromatase activity in the POA depends on androgenic stimulation. Conversion of testosterone to E_2 is markedly increased in castrated males injected intramuscularly (i.m.) with testosterone propionate (TP). Increased aromatase activity seems to be due to induction of the enzyme and is specific to the POA. These findings indicate that the formation of behaviourally effective oestrogen in a specific brain target area is under hormonal control and suggest a role for this regulatory mechanism in the integration of courtship behaviour in the male dove.

The brain system underlying nest-soliciting display in the male dove can be activated in short-term castrates by either testosterone or E_2 (ref. 7). However, while E_2 effectively restores nest-soliciting to pre-castration levels in long-term castrates, testosterone is virtually ineffective⁸, suggesting that capacity for aromatization declines with long-term androgen deficit. Prolonged treatment with TP increases nest-soliciting display to some extent in long-term castrates, suggesting that aromatase activity might increase due to the action of androgen⁸. Here we examine the possibility that androgen influences the preoptic aromatase system to increase the formation of E_2 .

In experiment 1, aromatization of testosterone was measured using a previously described microassay⁴. Two groups of birds ($n = 8$ for each group) were castrated and 30 days later they were injected on 12 successive days either with androgen or avian saline according to previously described procedures⁷. The dose of androgen (300 μg TP in 0.3 ml avian saline) used is known to induce nest-soliciting behaviour in 30-day castrates⁷. Males were tested daily for courtship⁷ and nest-soliciting behaviour was restored to pre-castration levels in the TP-treated group. Two brain areas were examined, the POA and a non-androgen target area, the area basalis (AB). Aromatase activity in the TP-treated group was increased about threefold as compared to control levels (Fig. 1a). This difference was highly significant ($P < 0.001$, Student's t -test). Aromatase activity was not affected in the AB, which is immediately adjacent to the POA but is not an androgen target area and shows much lower rates of testosterone conversion⁴.

To determine whether the effect of TP on aromatase activity is dose-dependent, a lower dose (30 μg per day) and shorter

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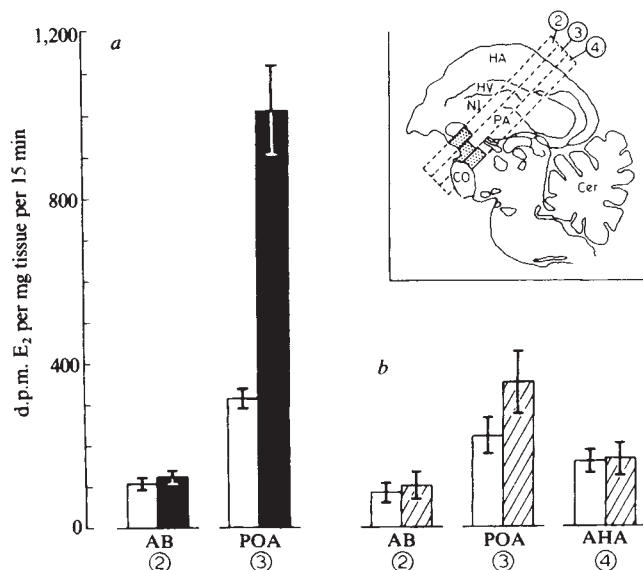


Fig. 1 *a*, Experiment 1: aromatase activity was markedly increased ($P < 0.001$, Student's *t*-test) in the POA of short-term castrates injected i.m. for 12 days with 300 µg per day of TP (solid bars) compared with saline-injected controls (open bars). Aromatase activity in the area basalis (AB) was not affected. *b*, Experiment 2: treatment with a lower dose of TP (30 µg per day) for 4 days (cross-hatched bars) was more effective than a single 30 µg injection (open bars) in increasing aromatase activity in the POA. It had no effect on either AB or the anterior hypothalamus (AHA); $n = 4$ in each group. Aromatase activity was measured in homogenates obtained from individual brain samples incubated at 40 °C, pH 7.4 for 15 min with 10 nM [1α , 2α (n)- 3 H] testosterone (specific activity 53 Ci mmol⁻¹, Radiochemical Centre, Amersham) and 1 mg ml⁻¹ NADPH₂ (Sigma, type III). Conversion rates were linear with respect to time and amount of brain tissue used for the incubation⁴. Inset: parasagittal view of the brain showing coronal sections 2, 3 and 4 from which samples AB, POA and AHA (stippled areas) were obtained. See ref. 18 for further details and nomenclature.

periods of treatment were used in experiment 2. In addition to AB and POA the anterior hypothalamus (AHA) was examined. This area is androgen-sensitive, but does not seem to concentrate E₂ to the same extent as the POA⁹. Aromatase activity was higher in the POA after 4 days of treatment than after a single injection (Fig. 1*b*), although the increase was not as great as that induced by the higher dose (expt 1). Again, this effect seems to be specific to the POA, no change being detectable in AB or AHA. The results of these experiments show that conversion of testosterone to E₂ is stimulated specifically in the POA of castrated male doves by systemic TP treatment. This effect is related to dose and duration of treatment.

In experiment 3, aromatase activity was measured in TP-treated (300 µg per day for 6 successive days) and untreated long-term castrates ($n = 4$ for each group) using increasing concentrations of the substrate (testosterone) to determine the kinetic properties of the enzyme. Figure 2 shows that the observed increase in activity is probably due to induction of the enzyme: the maximal velocity $V_{\max}$ was markedly increased (~fivefold: 250 fmol h⁻¹ per mg tissue as opposed to 48 fmol h⁻¹ per mg tissue) in the POA of castrates after six daily injections, but the Michaelis constant (K_m) was not significantly altered (2.1×10^{-8} M in controls as opposed to 2.7×10^{-8} M after TP treatment). Moreover, K_m in the AB was similar in either treated or untreated birds (2.1×10^{-8} M and 2.2×10^{-8} M, respectively), indicating that the same enzyme is present in both areas, although its tissue concentration is much higher in the POA.

These results indicate that the aromatase complex found in the dove brain has a high affinity for its natural substrate, testosterone and that it can be specifically induced in the POA by androgenic stimulation. An earlier study¹⁰ has suggested that

aromatase activity in the rabbit CNS may be influenced by hormonal condition. However, unlike our findings in the dove, aromatase activity was increased nonspecifically both by castration and systemic E₂ administration to intact male rabbits; testosterone had the same effect, but only when administered to intact females. The effect was not localized to a specific area of the brain known to be involved in mediating hormone action on a well-defined biological response, and there was no evidence for induction of the enzyme. Here we have demonstrated a specific induction by androgen on aromatase activity in the POA, a target area known to be associated with oestrogen-dependent behavioural patterns in the male dove. At this stage, we cannot exclude the possibility that systemically administered testosterone is acting via metabolites formed peripherally or centrally. The enzyme could be induced by E₂ itself, because basal rates of aromatization occur in the castrate brain in the absence of androgenic stimulation (Fig. 1). Irrespective of whether testosterone or a metabolite derived from it induces the enzyme, the effect is likely to involve both binding of the active steroid to a specific 'receptor' and genomic activation resulting in *de novo* synthesis of the aromatase complex.

The active steroid may, however, have an indirect action. Aromatase activity is stimulated in cultured brain cells¹¹ and choriocarcinoma cells¹² by cyclic AMP, and follicle-stimulating hormone (FSH) is a well-known regulator of gonadal aromatase

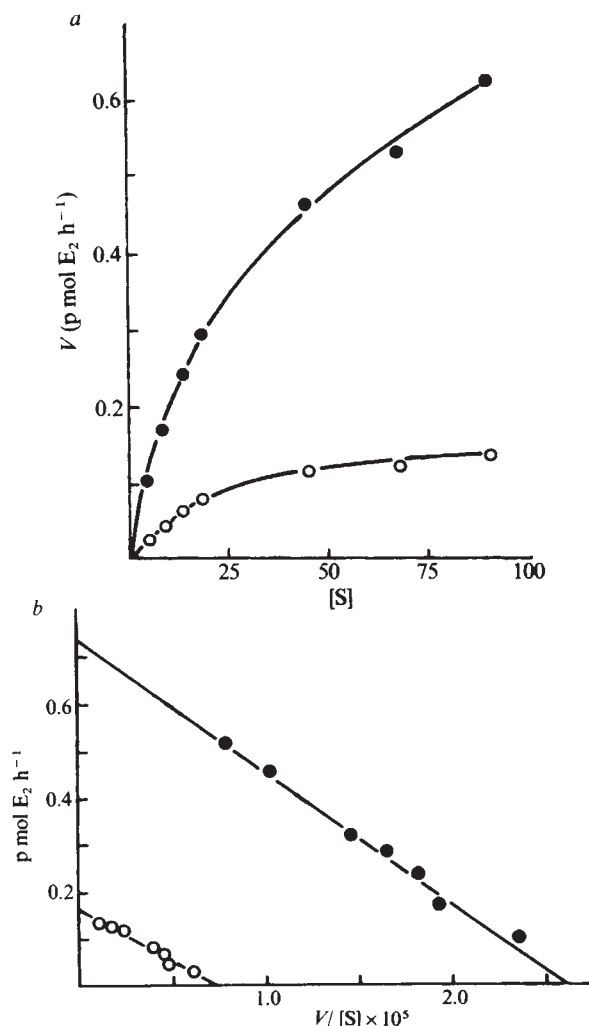


Fig. 2 *a*, Rate (V) against substrate concentration [S] (nM of testosterone) for aromatase activity in the POA of saline-treated (○) or TP-treated (300 µg per day for 6 days, ●) castrates. *b*, Eadie-Hofstee plot of the above data, from which the following values were calculated for the apparent kinetic parameters using unweighted linear regression. Saline-treated: $K_m = 2.75 \times 10^{-8}$ M, $V_{\max} = 250$ fmol h⁻¹ per mg tissue; androgen-stimulated: $K_m = 2.04 \times 10^{-8}$ M, $V_{\max} = 48$ fmol h⁻¹ per mg tissue.

activity¹³. Moreover, it has been shown recently¹⁴, using Sertoli cell-enriched cultures obtained from immature rats, that some adrenergic neurotransmitter agonists, notably L-isoprenaline and noradrenaline, are as effective as FSH or dibutyl cyclic AMP in stimulating conversion of testosterone to E₂ (ref. 14). Therefore, testosterone or its active metabolite could act by altering neurotransmitter levels in the POA. Further studies are now being carried out to elucidate the exact mechanism by which systemic TP treatment affects aromatase activity in the dove POA.

We suggest that aromatase activity and the production of behaviourally effective oestrogen in the dove POA are modulated by circulating androgens. Because there is evidence for oestrogen-sensitive mechanisms associated with behaviour in the male dove, this finding is likely to be of physiological significance. Thus, oestrogen acting within the brain induces a transition from predominantly aggressive to nest-oriented

courtship interactions with the female¹⁵. Plasma levels of testosterone reach a peak at the stage of the reproductive cycle when the behavioural transition normally occurs¹⁶. Increased aromatase activity in the POA induced by the testosterone peak may therefore mediate this transition. Moreover, oestrogen derived from testosterone in the POA, in addition to its specific effects on nest-orientated courtship, may also play a part in the regulation of testosterone dependent brain mechanisms of behaviour¹⁷. Therefore, the regulatory action exerted by androgen on aromatase activity in the POA is likely to have more than one effect on brain mechanisms involved in the integration of behaviour during courtship in the male dove.

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Benzodiazepines reduce stress-augmented increase in rat urine monoamine oxidase inhibitor

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Normal human urine inhibits monoamine oxidase (MAO)¹. This inhibitory activity, which is also present in rat urine, cannot be accounted for by a large range of known urinary constituents, including certain monoamines or their metabolites, but is caused by an unidentified low-molecular-weight compound(s). Endogenous MAO inhibitors may be important as physiological regulators; there have been reports of a decrease in platelet MAO activity, conceivably deriving from their presence, in a variety of human disease states². We show here that cold immobilization stress markedly increases the output of rat urine MAO inhibitor, and that this increase is attenuated by benzodiazepine drugs.

Figure 1 shows that control urine samples from unstressed rats produced comparatively little MAO inhibition. Cold immobilization stress increased fivefold the degree of MAO inhibition induced by rat urine, with no concomitant effects on urinary pH or creatinine concentration. Pretreatment of rats with lorazepam [0.25 and 1.25 mg per kg intraperitoneally (i.p.)] gave rise to a statistically significant decrease in the concentration of stress-induced urinary MAO inhibitor. The output of inhibitor at a dosage of 1.25 mg per kg lorazepam was less than that at 0.25 mg per kg. Similarly, acute doses of chlordiazepoxide (5, 10 and 50 mg per kg) also significantly reduced the output which was less at higher dosage.

Acute administration of benzodiazepines causes sedation whereas, after chronic administration, tolerance develops. Output of inhibitor was also examined, therefore, after 5 days' administration of 10 and 50 mg per kg chlordiazepoxide and found to remain significantly less than that of the stressed group

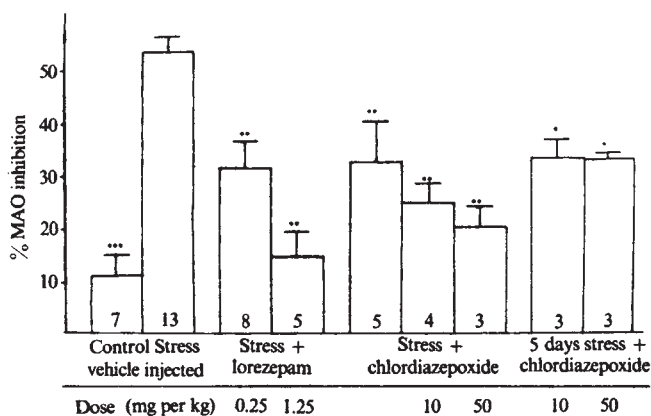


Fig. 1 Effect of cold stress in rats, with and without various benzodiazepine dosage schedules, compared with control animals. Values are means $\pm$ s.e. No. of animals is given at the base of each bar. *** $P < 0.001$ compared with vehicle-injected stressed group (2-tailed Student's *t*-test). ** $P < 0.01$, different from vehicle-injected stressed group (Neuman-Keul's test). * $P < 0.05$, different from vehicle-injected stressed group (Neuman-Keul's test). The data from the stressed group were subjected to analysis of variance and showed a significant drug effect ($F = 8.76$, $d.f. = 7.35$); $P < 0.0001$. The test system consisted of 100 μ l phosphate buffer (100 mM, pH 7.4), 20 μ l of urine, 20 μ l MAO preparation and 20 μ l ¹⁴C-tyramine, final concentration 83 μ M (for assay system, see ref. 1). All assays were carried out blind and in duplicate. Male hooded rats (Olac UK) (250–350 g) were starved overnight but allowed water. Controls were subjected to minimal handling and allowed to move freely in their cages. Stress was induced by placing rats in a restraint cage for 2 h at 4°C. Rats were decapitated with a guillotine and urine removed from the bladder by direct puncture. The urine was frozen and stored at –20°C before assay. Drugs were given by i.p. injection immediately before restraint. Chlordiazepoxide hydrochloride (Roche) was dissolved in distilled water to various concentrations to give an injection volume of 2 ml per kg. Lorazepam (John Wyeth) was dissolved in a vehicle of 2% benzyl alcohol, 18% polyethylene glycol and 80% propylene glycol in a concentration of 4 mg ml⁻¹. It was then diluted with distilled water to give injection volumes of 2 ml per kg. Stressed but drug-free animals received equivalent vehicle or water injections. For the chronic experiments, injections were given once per day for 5 days.

Table 1 Effect of stress and benzodiazepines on pH, creatinine levels and total bladder urine

	pH	Creatinine (mmol l ⁻¹)	Total bladder urine (mg)
Unstressed control (7)	6.6 ± 0.1	4.6 ± 1.2	199 ± 30
Stressed:			
Vehicle-injected (13)	6.5 ± 0.1	3.4 ± 0.5	154 ± 30
Lorazepam (0.25 mg per kg) (8)	6.4 ± 0.1	2.5 ± 0.4	107 ± 10
Lorazepam (1.25 mg per kg) (5)	6.6 ± 0.2	3.4 ± 1.0	169 ± 44
Chlordiazepoxide (5 mg per kg) (5)	6.6 ± 0.2	2.1 ± 0.7	103 ± 13
Chlordiazepoxide (10 mg per kg) (8)	6.5 ± 0.2	2.5 ± 0.8	125 ± 18
Chlordiazepoxide (50 mg per kg) (3)	6.4 ± 0.2	2.5 ± 0.7	131 ± 31
Chlordiazepoxide (10 mg per kg × 5 days) (3)	6.8 ± 0.2	4.9 ± 0.8	159 ± 18
Chlordiazepoxide (50 mg per kg × 5 days) (3)	6.6 ± 0.2	4.8 ± 0.6	164 ± 35

Numbers in parentheses indicate number of urine samples pooled.

(Fig. 1). Given acutely, chlordiazepoxide at 10 and 50 mg per kg caused sedation to 58% and 6% of control levels on an activity scale, whereas after 5 days the levels were 89% and 51% respectively³. These effects do not seem to correlate with inhibitor output, which remained significantly reduced after 5 days of 10 mg per kg chlordiazepoxide, suggesting that the benzodiazepine effect was not due solely to sedation.

Inhibitory potency was measured in a standard volume of urine. However, there was no significant difference in urinary pH, urine mass or creatinine concentration between the stressed and control groups, nor between stressed and drug-treated groups (Table 1). There was no correlation between any of these values and output of urinary MAO inhibitor. It therefore seems most unlikely that a difference in urinary flow could account for these findings.

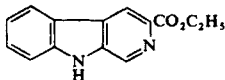
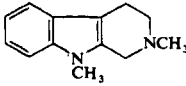
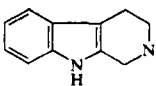
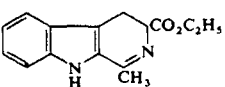
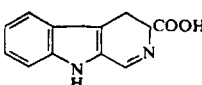
Ethyl acetate extracts of acidified pooled urine samples produced results similar to those produced by unextracted urine; the stressed group exhibited more inhibition than control, with intermediate values in benzodiazepine-treated groups, but with an approximate doubling of percentage inhibition in each (Table 2). This extraction behaviour points to a compound(s) of acidic or neutral nature and indicates that it is not a monoamine. The inhibitor is not generated spontaneously; activity remains constant when fresh rat urine is left at room temperature for 24 h.

Table 2 MAO inhibition by ethyl acetate extracts

	% Inhibition of MAO Pooled urine	Ethyl acetate extract
Unstressed control (7)	17.4	47.1
Stress vehicle-injected (13)	48.2	72.4
Lorazepam (13)	29.9	63.4
Chlordiazepoxide (12)	33.9	62.3

Numbers in parentheses denote number of urine samples pooled. Controls for this experiment consisted of four Wistar and three hooded rats. Equal volumes of urine from each rat in the stress and acute drug-treated groups in Table 1 were pooled. The urinary inhibitor was extracted into ethyl acetate as follows: pooled urine samples were diluted 1 in 10 with saturated NaCl acidified to pH 0.9 with HCl. This was shaken with an equal volume of ethyl acetate and centrifuged to separate the layers. The upper layer was aspirated off, the ethyl acetate removed by passing nitrogen through it and the remaining solids taken up to the original urinary volume with 100 mM phosphate buffer, pH 7.4. Blanks, in which water replaced urine, gave no MAO inhibition.

Table 3 MAO inhibition by carbolines

Drug	Structure	% MAO inhibition			
		10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶ M
Ethyl β -carboline-3-carboxylate (FG 7098)		27.3	13.7	6.0	0.7
2,9-Dimethyl 1,2,3,4-tetrahydro- β -carboline		66.8	54.0	35.4	14.3
1,2,3,4-Tetrahydro- β -carboline		45.6	21.1	7.8	0
Ethyl 3,4-dihydro-1-methyl- β -carboline-3-carboxylate		29.4	14.2	0	0
3,4-Dihydro- β -carboline-3-carboxylic acid		32.4	26.5	24.3	0.8

The drugs were dissolved in distilled water and the pH adjusted to 7.0. 100 μ l of each concentration was used in the reaction mixture instead of urine.

The differing patterns of output between the groups investigated seems unlikely to reflect any variation in dietary intake, for all urine samples were obtained after an 18–22-h fast. Nor is it likely to mirror any differences in gut flora; we have found previously that the excretion of urinary inhibitor is similar in control and germ-free rats¹.

It is possible that this urinary inhibitor derives from catecholamines or corticosteroids, the output of both of which is increased in this type of stress⁴. However, we have previously shown that none of the catecholamines or their major metabolites is present in human urine in sufficient concentration to account for its MAO inhibitory properties¹. In addition, there is no evidence that corticosteroids or their derivatives can inhibit MAO. We have, therefore, speculated about its possible identity in terms of a third chemical system involved in stress, possibly related to the β -carboline group of compounds⁵. Barker *et al.*⁶ have recently detected three tetrahydro- β -carbolines as normal constituents of rat brain and have also found particularly high concentrations in the adrenal gland. The molecular weight of the urinary inhibitor is of the appropriate order of 200 and carbolines such as harmine and harmaline are very potent MAO inhibitors⁷. Braestrup *et al.*⁸ have extracted a β -carboline from human urine which is a high-affinity ligand for benzodiazepine receptors. We have, therefore, tested Braestrup's compound (FG 7098) and four other structurally related compounds and found that they inhibit MAO in our test system only at concentrations much higher than the 10⁻⁸ M range in which FG 7098 was found in human urine (Table 3). However, Braestrup *et al.* indicate that the ester group of their compound was probably an artefact of the extraction procedure. As slight alterations in structure may markedly affect pharmacological properties, it remains possible that the MAO inhibitor or inhibitors we are studying are related to this compound or are endogenous compounds with a similar range of pharmacological actions.

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Modulation of affinity of postsynaptic serotonin receptors by antidepressant drugs

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Antidepressant drugs are generally thought to act by changing aminergic neurotransmission. Thus, according to the indoleamine hypothesis¹, antidepressant drugs increase the amount of serotonin (5-HT) available at the receptor level by inhibition either of 5-HT uptake or of its degradative enzyme (monoamine oxidase)². Various experimental observations have, however, suggested that the effects of antidepressant drugs may be located at the postsynaptic receptor level^{3–7}. We report here that antidepressant drugs alter the structural conformation of the postsynaptic serotonergic receptor in synaptosomal membranes isolated from rat and horse brains in such a way as to inactivate them.

Serotonin postsynaptic receptors are regulated by a mechanism involving several conformational states of the recognition site for 5-HT⁸, with different affinities for 5-HT. The transition of the receptor protein from one state to another modulates the activity of the effector system, a 5-HT-sensitive adenylate cyclase^{9,10}. In the resting state, the recognition site binds 5-HT with a low affinity. Exposure to an agonist (but not to an antagonist) induces the structural transition of the receptor to an intermediate state stabilized by GTP; this state corresponds to the activation of the adenylate cyclase. The transition process ends with a final high-affinity state which can no longer activate the effector (desensitized state).

Our data consist of measurements of the affinity of ³H-5-HT for synaptic membranes pre-treated with various drugs (Fig. 1). Pre-exposure of the membranes to imipramine (10 nM) changed the affinity of the receptor for 5-HT; the affinity constant, K_D , was ~12 nM in the control and ~2 nM after pre-exposure to the antidepressant (Fig. 1). This effect is comparable with that observed after pre-exposure of the membranes to 5-HT itself (Fig. 1). Imipramine is also effective at a lower concentration (1 nM) where a similar increase in affinity is observed (Fig. 2a) and in this case, the total number of binding sites is almost unchanged (<15%). The drug also produced an increase in affinity at a higher concentration (0.1 μ M), but there was a marked reduction in the number of sites binding ³H-5-HT (40–60%). Note that this effect is not the result of a direct binding of the imipramine to the serotonergic site as the K_I (10 μ M) of the drug (measured using imipramine concentrations which competitively inhibit ³H-5-HT binding) is several orders of magnitude greater than those which produce an increase in affinity. Furthermore, the presence of a competitive inhibitor would be expected to decrease the apparent affinity, not increase it as seen here.

The affinity change induced by imipramine is not observed in the presence of GTP (10⁻⁴ M, Fig. 2b). Other tricyclic antidepressant drugs have also been tested with similar results (see Table 1). Other non-antidepressant substances tested, chlorpromazine (10 nM and 10 μ M, Fig. 2b), antiserotonins, cinan-

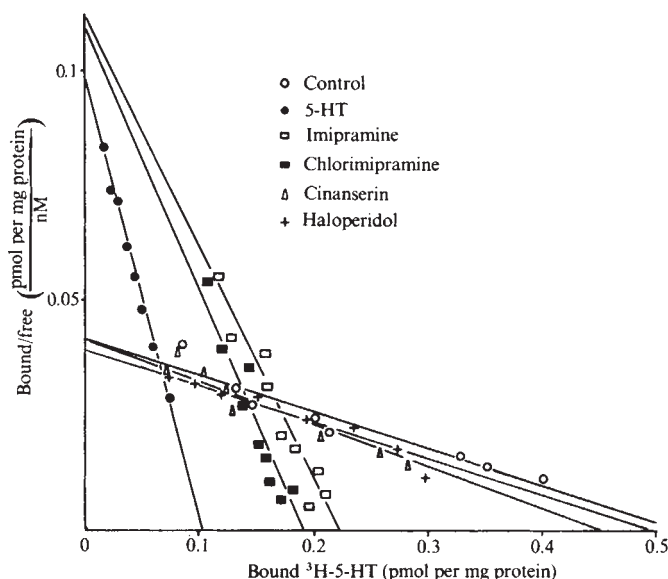


Fig. 1 Scatchard plot of ³H-5-HT binding to synaptic membranes pre-exposed to various drugs. Assays were done using membranes isolated from rat brains. Sprague-Dawley rats were decapitated and their brains isolated on ice and the whole forebrain dissected. These tissues were homogenized and treated as described elsewhere¹² to obtain enriched preparations of synaptosomal membranes by differential and density gradient centrifugation. Synaptosomal membranes were preincubated at 37 °C for 10 min to eliminate the endogenous 5-HT which could still be present and which could interfere with the binding of ³H-5-HT as shown previously^{18,19}. These were then diluted 10 times in Tris-HCl buffer, 50 μ M, pH 7.4, containing nialamide (100 μ M) and ascorbic acid (0.1%). The membranes were then pre-exposed to 5-HT (10 nM) at 37 °C for 10 min or 15 min or to the substance being investigated at the indicated concentration (generally 10 nM); controls were incubated in buffer. The samples were washed either by centrifugation (20,000g, 10 min) and dilution in the initial volume of buffer, or by simple dilution (20-fold). Aliquots of these washed membranes were incubated at 22 °C for 8 min in the presence of various concentrations of ³H-5-HT (2–30 nM). Specific binding was determined as previously described using a rapid filtration technique. Results for membranes isolated from striatum or hippocampus of horse brain were not significantly different. In the experiment shown, rat brain membranes isolated from striatum were pre-exposed to buffer or 10 nM of the indicated drug and washed. After incubation in the presence of ³H-5-HT, the K_D observed after preincubation in buffer, 5-HT, imipramine, chlorimipramine, cinanserin and haloperidol was 12.4, 1.07, 2.08, 1.56, 12.7 and 13.5 nM, respectively.

serin, methysergide, methergoline, methiothepin (10 nM), neuroleptics, haloperidol (10 nM) and fluphenazine (10 nM) had no effect.

Interestingly, the change in affinity induced by 5-HT is inhibited by methysergide (10 μ M) added together with the amine during pre-exposure of the membranes; a similar inhibition of the effect of imipramine is observed in the same experimental conditions.

The change in the affinity for ³H-5-HT induced by pre-exposure of the membranes to 5-HT itself occurs in rat brain synaptosomal preparations and in membranes isolated from striatum⁸ or from hippocampus. This phenomenon seems to be widespread for serotonergic sites as we also observed it in membranes isolated from horse brain (striatum and hippocampus). As we have previously shown¹¹, these sites are located on postsynaptic membranes. The affinity constant observed for 5-HT binding to membranes which had not been pre-exposed is ~15 nM, whereas membranes pre-exposed to 5-HT and washed bind 5-HT with a higher affinity ($K_D = 1.8 \pm 0.4$ nM). This increase in affinity is induced by serotonergic agonists and not by antagonists. As previously reported^{9,10}, these affinity changes might correspond to structural changes of the receptor protein as they do not occur after pretreatment of the membranes with a sulphhydryl group reagent.

In experimental conditions which produce a high-affinity binding for 5-HT, no adenylate cyclase activation is observed, whereas in the presence of GTP (or GppNHp) the low-affinity binding is stabilized and adenylate cyclase activation occurs^{9,10}. These observations and other information on binding kinetics

Table 1 Affinity constant of ^3H -5-HT binding to synaptic membranes

Pre-exposure	K_D (nM)
None	15.3 ± 1.26 (14)
Serotonergic agonists	
5-HT	1.8 ± 0.42 (8)
Bufotenine	3.23
5-MeO-N,N-dimethyltryptamine	2.08
Tricyclic antidepressants	
Imipramine	2.42 ± 0.22 (6)
Chlorimipramine	1.72 ± 0.99 (3)
Amitriptyline	4.2 ± 0.35 (3)
Trimipramine	4.0 ± 0.3 (3)
Desipramine	4.9 ± 0.69 (3)
Doxepin	4.5
Atypical antidepressants	
Iprindole	3.9 ± 1.02 (3)
Mianserine	2.9 ± 0.88 (3)
Fluoxetine	2.45 ± 0.22 (3)
Fenfluramine	2.1
Serotonergic antagonists	
Methysergide	12.43 ± 0.48 (3)
Methergoline	9.8
Cinanserin	12.7
Methiothepin	15.0 ± 2.9 (3)
Other drugs	
Chlorpromazine	14.24 ± 1.95 (5)
Fluphenazine	10.7 ± 1.9 (3)
Haloperidol	13.5

Affinity changes of ^3H -5-HT binding induced by various drugs. Membranes were pre-exposed to the drug being investigated (10 nM) at 37°C for 10 min, diluted 20-fold in Tris-HCl buffer (50 mM, pH 7.4) and incubated at 22°C for 8 min, in the presence of various concentrations of ^3H -5-HT. The radioactivity specifically bound was determined by a rapid filtration technique. The affinity constants are the means $\pm$ s.e. of 3–6 assays. For the drugs tested twice only, the values observed for individual assays did not differ by $>30\%$ of the mean.

led us to propose the regulatory mechanism summarized above.

All the drugs tested which have clinical properties of antidepressants, except the group of the inhibitors of monoamine oxidase (MAO), are able to increase the affinity of the 5-HT receptor for the amine itself. Active drugs include tricyclic antidepressants (imipramine, chlorimipramine, amitriptyline, trimipramine, desipramine) which are also inhibitors of 5-HT or noradrenaline (NA) uptake, other antidepressants which are only weak inhibitors of amine uptake (iprindol or mianserin) and doxepin which is considered to be a weak antidepressant. The changes in affinity induced by these drugs do not therefore seem to be related to their capacity to inhibit the uptake of 5-HT or NA. Furthermore, many of the drugs, are used at concentrations which have no effect on the monoamine uptake¹². The clinical properties of fluoxetine and fenfluramine as antidepressants have not been established.

Note that the antidepressants of the MAO group are ineffective. They are known to act on the degradative enzyme of 5-HT, resulting in an increase in the availability of 5-HT in the synaptic cleft. These drugs do not increase the affinity of the binding site for ^3H -5-HT. The assay could be performed in the presence or absence of 100 μM nialamide without any alteration to the ability of the binding site to be either in a conformational state corresponding to a high or a low affinity for ^3H -5-HT.

Chlorpromazine, which is not considered to possess true antidepressant properties although it has a chemical structure very like that of imipramine, is unable to increase the affinity of the 5-HT receptor for its ligand even at concentrations up to 10^{-6} M; haloperidol and fluphenazine, neuroleptic drugs which lack antidepressant properties, are also ineffective.

Thus, this effect of the antidepressant drugs on the post-synaptic serotonergic receptor seems to be specific. It does not occur directly at the serotonergic site by competition with 5-HT as the affinity constants of these drugs for the 5-HT site correspond generally to a rather low affinity in the range 1–10 μM ¹³. Several hypotheses might be proposed to explain this effect. The antidepressant drugs might act by binding to a specific site corresponding to the existence of an endogenous imipramine-like ligand. In that case, the occupation of the site would modulate the conformation of the serotonergic recep-

tors possibly through an allosteric process. Binding sites for ^3H -imipramine have already been described in synaptosomal membranes from rat brain¹⁴. On the other hand, these drugs might act by changing some physical characteristics of the membranes in a manner analogous to that described by Hirata and Axelrod¹⁵ in the close vicinity of the serotonergic receptor.

The drugs which induce the transition of the site to high affinity are either 5-HT agonists or antidepressants. We have shown that the binding of 5-HT to this conformation of the site is unable to activate the adenylate cyclase and therefore produces a desensitized state of the receptors; antidepressant drugs thus

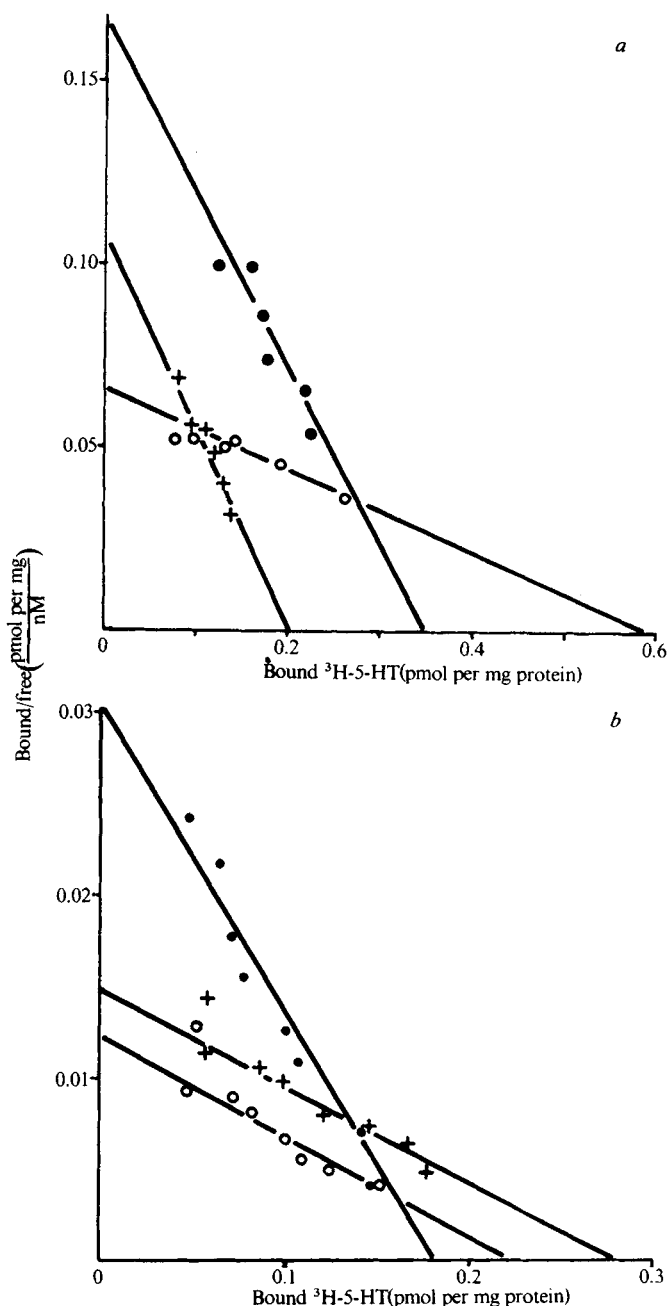


Fig. 2 *a*, Effect of the pre-exposure of membranes to imipramine on the binding of ^3H -5-HT to its specific sites. Scatchard plot of the binding curves for membranes not pre-exposed to imipramine (control, ○); and pre-exposed to 1 nM (+) and 10 nM (●) imipramine. Experimental conditions were the same as for Fig. 1. The affinity constants for ^3H -5-HT were 11, 2.2 and 2.04 nM for control, 1 and 10 nM imipramine, respectively. *b*, Lack of effect of imipramine pre-exposure on purified synaptosome membranes treated with GTP. The same experimental conditions were used as for Fig. 1 except that the test membrane preparation contained GTP during pre-exposure to imipramine. The corresponding affinity constants were: $K_D = 5$, 19.1 and 17.2 nM after pre-exposure of the membranes to buffer (control, ○), imipramine (10 nM, ●) and imipramine + GTP (10 μM , +), respectively.

seem to inactivate the postsynaptic 5-HT receptor. Because drugs which lack antidepressant properties do not have this capacity, we suggest that the clinical properties of tricyclic antidepressants are related to their capacity to desensitize the postsynaptic 5-HT receptor.

The structural changes induced by tricyclic antidepressants might represent a critical step in the receptor modifications observed after long-term administration^{7,16,17}. Our results support new concepts of the mechanism of action of antidepressants and indicate that the clinical properties of these drugs might involve an effect on the postsynaptic 5-HT receptor.

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Transplanted adrenal chromaffin cells in rat brain reduce lesion-induced rotational behaviour

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Rats with unilateral lesions of substantia nigra pars compacta (SN), the area of the brain containing most dopamine-containing neurones, are a widely recognized animal model of Parkinson's disease^{1–4}. When given dopamine agonists such as apomorphine, such rats rotate in a direction contralateral to the lesion, presumably because of the development of supersensitive dopamine receptors in the striatum ipsilateral to the lesion. When grafts of embryonic SN are placed in the lateral ventricle^{5,6}, or into a transplant cavity⁷ adjacent to the striatum in animals with SN lesions, this rotational behaviour has been shown to decrease. Histochemical examinations have shown that axons from the grafts have grown into the striatum^{8–7}, and biochemical measurements indicate that dopamine concentrations are increased in areas of the striatum adjacent to the SN grafts⁶. Nevertheless, an obvious problem with this technique, both for basic research and possible clinical applications, is the requirement for fetal central nervous donor tissue. We now describe how grafts of adrenal medulla can be used with similar effects, involving chromaffin cells.

There are several reasons for considering the adrenal medulla as a potential replacement for fetal SN grafts. First, the normal

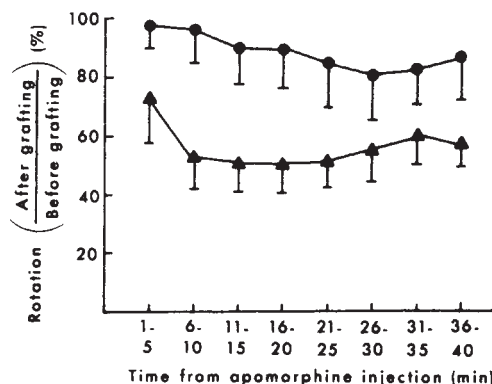


Fig. 1 Contralateral apomorphine-induced turning after grafting, expressed as a percentage of turning before grafting, is shown as a function of time after apomorphine injections. To derive these data, the 40-min testing sessions were divided into eight 5-min segments. For each of these segments, a mean pregrafting rotation rate was determined, and the rotation rate after grafting was expressed as a percentage of this pregrafting rotation rate. Triangles indicate animals that received adrenal medulla grafts ($n = 13$); circles indicate control animals that received sciatic nerve grafts ($n = 15$). Vertical lines indicate s.e.m. A two-way analysis of variance for one repeated measure showed a significant main effect of treatment (adrenal versus sciatic nerve grafts [$F(91,26) = 4.40$; $P = 0.043$]). The main effect of measures and measures $\times$ treatment interactions were not significant ($F(7,182) = 1.71$; $P = 0.109$) and ($F(7,182) = 0.81$; $P = 0.417$, respectively).

adrenal medulla produces dopamine as an intermediary in the synthesis of adrenaline^{8,9}. Second, adrenal chromaffin cells, which are normally rounded in shape, become angular and develop processes when grown as grafts in the anterior eye chamber^{10–12} or when grown in culture in the absence of corticosteroids^{13–15}. Finally, processes originating from intraocular adrenal medulla grafts can innervate intraocular grafts of cerebral cortex, as demonstrated by histochemical studies¹⁶.

Unilateral SN lesions were produced^{5,6} in male Sprague-Dawley rats by stereotaxic injections of 6-hydroxydopamine hydrobromide into the right SN. Approximately 2 months later, the animals were tested for apomorphine-induced (0.1 and 0.25 mg per kg subcutaneously) rotational behaviour using an automated apparatus. The smaller of the two doses that produced at least 80 counterclockwise rotations in 40 min was used for each animal. Animals turning at less than this rate were not used. After the initial screening, rotational behaviour was re-examined for five to seven additional sessions to obtain a stable baseline for each rat.

Adrenal glands were removed from young adult Sprague-Dawley rats (125–200 g) and the medulla dissected free. The dissected medulla was then bathed in sterile lactated Ringers at room temperature. A cut was made in the surface of the medulla to enable it to be opened, and the interior was removed. This method was used to avoid inclusion of the adrenal cortex in the graft. Four to six grafts were implanted in the lateral ventricle on the 6-hydroxydopamine-lesioned side in each of 13 rats (age at least 5 months) using Pellegrino and Cushman¹⁷ coordinates of 1.5 mm lateral and 1.5 anterior to bregma, 3.5–4.0 mm below the dura, as previously described. Control ($n = 15$) animals received grafts of sciatic nerve instead of adrenal medulla.

Adrenal medulla ($n = 13$) or sciatic nerve (control) grafts ($n = 15$) were implanted and 2 months allowed for survival and growth of the graft tissue. At this time, rotational behaviour was examined for five 40-min sessions, and some of the animals killed for histochemical studies.

When the rats were tested 2 months after transplantation, rotational behaviour was significantly less in the animals with adrenal medulla grafts than in those with sciatic nerve grafts (Fig. 1). Histochemical fluorescence^{18–20} studies of six randomly selected rats showed the denervations of the caudate nucleus to

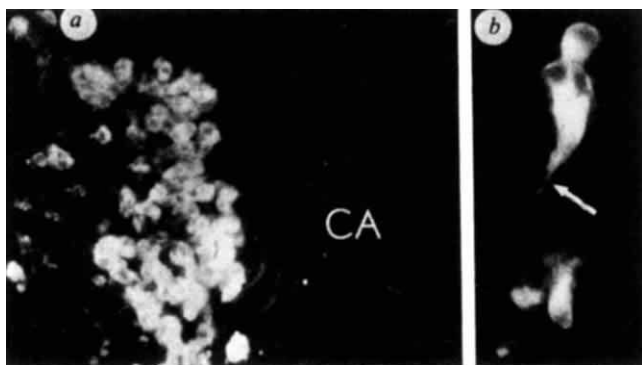


Fig. 2 Falck-Hillarp fluorescence microscopy of intraventricular adrenal medullary grafts. *a*, Overview of cluster of strongly fluorescent chromaffin cells within a graft. Some cells are elongated or polygonal with short coarse processes (upper left), but very few thin nerve fibre-like processes have formed (CA, caudate). $\times 160$. *b*, Close-up of two chromaffin cell clusters. Three nuclei are seen in the lower cluster: one cell has a tapering nerve fibre-like process (arrow). $\times 290$.

be essentially complete, with only a few fine terminals remaining in one rat. Grafts with catecholamine-containing chromaffin cells were found in five of the six animals. As shown in Fig. 2, these cells had developed polygonal shapes and were usually grouped in one or more small clusters within the grafts. Two types of fluorescent cells were found in the adrenal grafts, similar to the noradrenaline (very strongly fluorescent) and adrenaline (moderately fluorescent) cells present in normal adrenal medulla^{21,22}. Some of these cells had fine elongated processes, but very few fibres were seen to leave the grafts and enter the host brains. The total numbers of chromaffin cells found in the grafts counted by methods previously described³, were 880, 4,080, 66, 517 and 2,134, respectively, for the five animals. Adrenal cortex was also found in the graft that contained 4,080 chromaffin cells. Rotational behaviour was not reduced in this animal, nor in the animal that had only 66 chromaffin cells.

The observations suggest that adrenal medulla grafts can reduce lesion-induced rotational behaviour, even though there was no clear evidence that the grafts actually reinnervated the host caudate nucleus. Although the transplanted chromaffin cells were elongated and produced fine fibres, these fibres were found almost entirely within the grafts, and rarely penetrated into the caudate nucleus. This suggests that catecholamines or other substances diffused from the grafts to receptor sites in the caudate nucleus in sufficient quantity to reduce caudate dopaminergic supersensitivity and consequently, apomorphine-induced rotation.

Adult adrenal medullary cells show a remarkable phenotypic plasticity in response to environmental changes. Thus, when pieces of adrenal medulla are grafted into the anterior chamber of the eye, chromaffin cells will migrate into the sympathetically denervated host iris and form catecholamine-containing nerve fibres that reinnervate the iris¹⁰. Tissue culture experiments show that chromaffin cells of rodents and man express the endocrine phenotype in the presence of glucocorticoids and the sympathetic adrenergic neurone phenotype in the absence of glucocorticoids and the presence of nerve growth factor (NGF)^{12-15,23}. Thus, histochemical changes in grafted adrenal medullary tissue in the eye may be explained by the removal of the normal adrenal cortical environment and increased amounts of NGF in the sympathetically denervated host iris (see ref. 24). We have recently shown that chromaffin cells grafted into the eye chamber can also innervate adjacent grafts of brain tissue¹⁶. In this case, fine varicose fibres, typical of telencephalic adrenergic afferents, were found. In contrast to rat chromaffin cells, bovine chromaffin cells show little or no evidence of fibre

production when cultured or transplanted to the anterior eye chamber or brain^{15,25}.

Although the results reported here clearly demonstrate long-term survival of grafted chromaffin cells and reduction of apomorphine-induced rotational behaviour, there was only partial transformation of chromaffin cells towards the neuronal phenotype. Brain tissue does not seem to contain or produce any appreciable amount of NGF^{26,27}. Therefore, these results agree well with the findings from tissue culture that chromaffin cells will survive and produce small numbers of nerve fibres without NGF, but require NGF for extensive fibre formation (see refs. 15,23). Because of the sparsity of innervation of host caudate, the present behavioural results are probably best explained by a release of catecholamines from the grafts and subsequent diffusion of the amines to supersensitive dopamine receptors. Fluorescence microscopy demonstrated that both noradrenaline and adrenaline cells were present in at least some of the grafts, although the proportion of noradrenaline cells seemed higher in grafts than in normal adrenal medulla. The relative role of dopamine, noradrenaline and adrenaline and other possible neurotransmitters or peptides released by the grafts, such as enkephalin²⁸⁻³⁰, remains to be determined. Experiments are in progress to measure the concentrations of various catecholamines in the graft and adjacent host brain. Conceivably, release might also be influenced by any fibres from the host brain which innervate the grafted tissue, analogous to the normal regulation of medullary catecholamine release by cholinergic afferent input. A further question would be the presence of all-or-none action potentials and impulse-dependent transmitter release. Action potentials have been reported in the NGF-transformed phaeochromocytoma line PC-12 by Greene³¹.

Although many questions remain unanswered, the data reported here suggest that it may be possible to use adrenal medulla in place of fetal SN as a source of tissue for catecholamine-containing grafts. If further studies are successful, peripheral autografts could be potential replacements for destroyed or damaged central neurones. We are now attempting to produce adrenal autografts in subhuman primates and to develop protocols to increase fibre ingrowth from adrenal grafts to the caudate nucleus.

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Immunological enhancement of tumour allografts following treatment of mice with TNP-conjugated alloantigen and anti-TNP antibody

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Immunological enhancement—the prolonged survival of tumour or organ allografts brought about by passive immunization of the host with alloantibody directed against the graft's antigens—has not yet been applied successfully to clinical organ transplantation. There are many reasons for this, among them the difficulty of preparing the required battery of alloantisera and the danger of causing hyperacute rejection of kidney allografts. We show here that it should be possible to circumvent both these problems by using a xenogeneic antibody directed against a well defined hapten rather than against histocompatibility antigens.

The mechanism of immunological enhancement is still a matter for debate¹⁻⁵. One hypothesis advanced to account for antibody-mediated suppression of specific immune responses is that graft antigen and anti-graft antibody combine to form complexes which are bound specifically by the antigen receptors of lymphocytes, by virtue of free antigenic determinants. In this way, antigen-specific cells (ARCs) of the recipient are coated with antibody indirectly bound to the lymphocyte membrane through an antigen bridge. It is postulated that these opsonized ARCs are subsequently destroyed by Fc-receptor-bearing host cells such as macrophages⁶⁻⁹. The most direct evidence for the hypothesis of ARC opsonization (ARCO) (reviewed in ref. 4) comes from experiments in which syngeneic radiolabelled ARCs were taken up by the reticuloendothelial system when injected intravenously (i.v.) into rats bearing enhanced kidney transplants^{6,7} or mice bearing enhanced skin allografts¹⁰. This is strongly supported by experiments in which mice were pretreated with complexes between sheep red blood cells (SRBCs) and anti-SRBC antibody⁸ or with allogeneic cells and alloantibody⁹.

Following the ARCO hypothesis, one possible prediction is that the antigenic determinant in the complex bound by receptors of ARCs need not be identical with that reacting with the

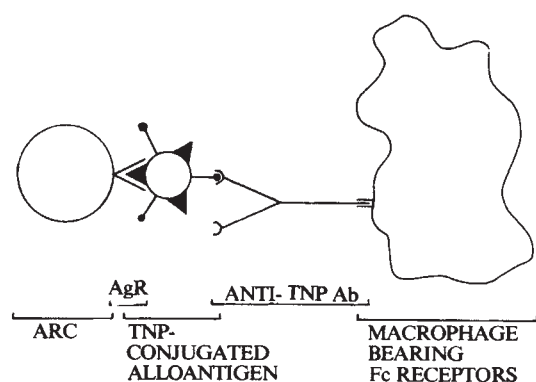


Fig. 1 The concept of opsonization of alloreactive ARCs by TNP-conjugated alloantigen and anti-TNP antibody. Free alloantigenic determinants (Δ), in complexes formed between TNP-conjugated alloantigens (where $\bullet$ signifies TNP determinant) and anti-TNP antibody, are specifically bound by the antigen receptor (AgR) on the antigen-reactive cells (ARCs). In this way ARCs are opsonized by antibody indirectly bound to the cell surface through an antigen bridge, to be destroyed by Fc-receptor-bearing host cells.

Table 1 a, Enhancement of P815 (H-2^d) tumour in A strain (H-2^a) mice by anti-H-2^d serum; b, enhancement of EL-4 (H-2^b) tumour in A strain mice by anti-H-2^b serum

Serum (μ l)	MTA $\pm$ s.e. (mm ²)	P
a None	28.0 $\pm$ 3.4	
10	66.4 $\pm$ 8.8	<0.01
25	84.0 $\pm$ 11.4	<0.01
50	58.6 $\pm$ 7.0	<0.01
100	40.7 $\pm$ 3.0	<0.05
b None	34.7 $\pm$ 1.1	
12.5	69.2 $\pm$ 7.7	<0.01
25	87.5 $\pm$ 9.0	<0.001
50	76.1 $\pm$ 11.6	<0.01

MTA, mean tumour area estimated on day 7. Values are for groups of six mice.

enhancing antibody (Fig. 1). It has indeed been shown that, to achieve the most efficient opsonization of ARCs, the ARC receptors and the antibody should bind to different determinants in the complex⁹, thus avoiding blocking of the determinants required for the linkage to cell receptors. Sinclair and Law¹¹ have recently shown that complexes formed between trinitrophenyl (TNP)-conjugated alloantigen and anti-TNP antibody can suppress *in vitro* alloimmune responses, and that anti-TNP antibody enhances the *in vivo* growth of TNP-conjugated allogeneic tumour cells. We too have found that alloantigens coupled to TNP retain alloantigenic activity, that they can be specifically bound by alloreactive ARCs, and that ARCs which have bound TNP-alloantigen/anti-TNP antibody complexes are specifically diverted to the liver when injected i.v. into normal mice¹². Our present experiments confirm that anti-TNP antibody enhances TNP-conjugated tumour allografts and we have extended these observations by showing that complexes between TNP-conjugated alloantigen and anti-TNP alloantibody specifically enhance the growth of unmodified tumour cells.

The mice used were from inbred strains BALB/c (H-2^d), DBA/2 (H-2^d), C57BL/6 (H-2^b) and A/J (H-2^a) bred by the Animal Department of St Mary's Hospital Medical School, London. They were 6–10 weeks old at the start of each experiment. Anti-H-2^d and anti-H-2^b alloantisera were raised by injecting five doses of 2×10^7 allogeneic (BALB/c or C57BL/6) spleen cells intraperitoneally (i.p.) into female A strain mice at 2-week intervals. Anti-TNP serum was produced in female New Zealand White rabbits by the injection of 500 μ g TNP-KLH (keyhole limpet haemocyanin) antigen in complete Freund's adjuvant on days 0 and 21, followed by i.v. booster inoculi of 1 mg of the same antigen in saline on days 45 and 59. The rabbits were bled on day 70 and the freshly prepared sera were stored at -20°C . P815 mastocytoma and EL-4 leukaemia cells were maintained by i.p. passage in DBA/2 and C57BL/6 mice, respectively. Conjugation of cells with TNP¹³ was achieved by incubating splenic lymphocytes or tumour cells at a concentration of 2.5×10^8 per ml in 10 mM trinitrobenzene sulphonic acid (TNBS, Sigma) dissolved in phosphate-buffered saline (PBS), pH 7.2, at 37°C for 10 min. The cells had been washed in PBS before incubation and were washed four times after incubation. The cell membrane antigen was prepared by a modification of the technique described by Standing and

Table 2 Enhancement of P815 tumour cells by TNP-BALB/c antigen and anti-TNP serum

TNP-BALB/c antigen (spleen cell equivalents)	Anti-TNP serum (ml)	MTA $\pm$ s.e. (mm ²)	P
None	None	33.1 $\pm$ 5.3	
3.2×10^7	0.32	81.5 $\pm$ 7.0	<0.001
10^7	0.1	59.1 $\pm$ 9.6	<0.05
3.2×10^6	0.032	53.1 $\pm$ 9.0	NS
10^6	0.01	51.6 $\pm$ 7.0	NS

MTA, mean tumour area estimated on day 7. Values are for groups of five mice. NS, not significant.

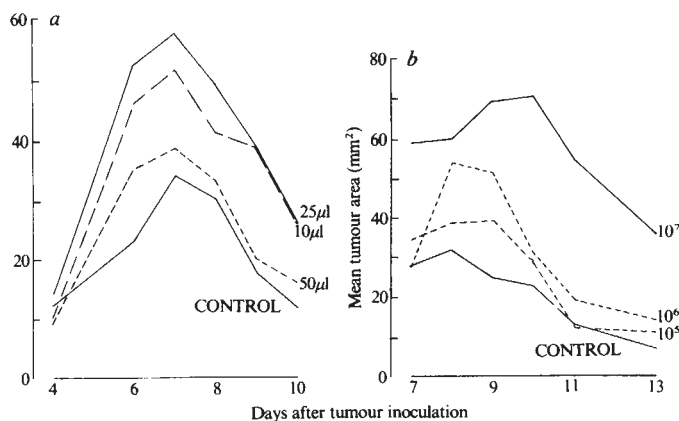


Fig. 2 *a*, Enhancement of TNP-conjugated tumour by anti-TNP serum. P815 mastocytoma cells (H-2^d) were hapten-conjugated by incubation in 10 mM TNBS (see text). 10⁷ viable TNP-P815 cells were injected s.c. into A strain (H-2^a) mice and 0–50 µl rabbit anti-TNP-KLH serum was injected i.p. Control mice received no serum. Tumour size was measured at intervals after inoculation and the mean tumour area (MTA) calculated as described in the text. *b*, Enhancement of tumour allografts by TNP-conjugated alloantigen and anti-TNP serum. 10⁷ viable P815 mastocytoma cells (H-2^d) were injected s.c. into A strain (H-2^a) mice. TNP-conjugated BALB/c (H-2^b) cell membrane antigen (see text), derived from 10⁵–10⁷ cells, was injected i.v. and 0.1 ml rabbit anti-TNP-KLH serum was given i.p., both at the time of tumour inoculation. Control mice received no treatment. Tumour size was measured on days 7–13 and the MTA calculated.

Williams¹⁴. Thus, TNP-conjugated or unmodified spleen cells were pelleted, resuspended in 2% Tween 40 (Sigma) in 0.1 M Tris-HCl buffered saline (pH 8.0) and stirred at 0 °C for 60 min. The cells were then homogenized by 20 strokes of a Dounce glass homogenizer and the suspension was centrifuged at 2,000g for 15 min. The supernatant was centrifuged again, this time at 100,000g for 45 min, and the sedimented membrane material was resuspended in PBS and stored in aliquots at –80 °C.

The experimental design was as follows. 10⁷ tumour cells in 0.1 ml balanced salt solution (BSS) containing 10% fetal calf serum (FCS) were injected subcutaneously (s.c.) into A strain mice on day 0. Different doses of antigen (0–10⁸ cell equivalents) and alloantisera or anti-TNP serum (0–0.5 ml) were injected i.v. and i.p., respectively, within 1 h of tumour cell inoculation. The resulting tumours were measured from day 6 onwards. The diameter (*d*) of the tumours was measured in two planes at right angles to each other and the tumour surface area (*A*) was calculated according to the formula: $A = \pi(d_1 \times d_2)/4$. The statistical significance of the data was assessed using Student's *t*-test.

A-anti-BALB/c (anti-H-2^d) serum enhanced the growth of the P815 (H-2^d) tumour in A strain (H-2^a) mice (Table 1*a*). The optimal effect was obtained using 25 µl per mouse; presumably, higher doses were cytotoxic to the grafted cells (see below). Similar results were obtained using the EL-4 (H-2^b) tumour cells and an anti-C57BL/6 (H-2^b) serum (Table 1*b*). The enhanced growth was clearly strain specific in that A-anti-BALB/c serum did not enhance the growth of EL-4 and anti-C57BL/6 serum did not enhance the growth of the P815 tumour (results not shown).

Similarly, TNP-antisera enhanced the growth of TNP-conjugated tumour cells (TNP-P815; Fig. 2*a*), thus confirming the work of Sinclair and Law¹¹. Again, 25 µl proved to be optimal although 10 µl were here almost as effective; 50 µl had no

significant effect. TNP-P815 cells are, in fact, readily lysed *in vitro* by anti-TNP serum and complement (I.V.H., unpublished), a finding that almost certainly accounts for the lack of success with the higher dose.

Figure 2*b* shows that unmodified P815 tumour implants were enhanced through the use of TNP-BALB/c membrane antigen and anti-TNP antibody. Strain A mice were given 10⁷ unmodified tumour cells s.c., 0.1 ml anti-TNP serum i.p., and TNP-modified cell membrane antigen at 10⁵–10⁷ cell equivalents per mouse i.v. Tumour growth was considerably enhanced in animals treated with TNP-conjugated membranes obtained from 10⁷ cells and with 0.1 ml antiserum, whereas doses representing 10⁵ or 10⁶ cells were less effective. When the dose of antigen and antiserum was increased whilst maintaining the ratio of 10⁷ cell equivalents to 0.1 ml serum, the level of enhancement was correspondingly greater (Table 2). TNP-BALB/c cell membrane alone, at doses of 10⁵–10⁸ cell equivalents, had no effect (Table 3). Finally, it was shown that the enhanced state was strain specific. Strain A mice treated with TNP-BALB/c (H-2^d) or TNP-C57BL/6 (H-2^b) cell membrane antigen and anti-TNP serum were inoculated with P815 (H-2^d) or EL-4 (H-2^b) tumour cells. TNP-BALB/c membrane enhanced P815 but not EL-4 and, conversely, TNP-C57BL/6 membrane enhanced EL-4 but not P815 (Table 4).

The data therefore show that anti-TNP antibody enhances the growth of TNP-conjugated tumour cells, thus confirming the finding by Sinclair and Law¹¹, and further, that it enhances the

Table 4 Specificity of tumour enhancement by TNP-conjugated antigen and anti-TNP serum

Treatment	P815 tumour		EL-4 tumour	
	MTA ± s.e. (mm ²)	<i>P</i>	MTA ± s.e. (mm ²)	<i>P</i>
Control	16.9 ± 2.2		34.7 ± 1.1	
TNP-BALB/c antigen	18.4 ± 2.8	NS	40.3 ± 4.9	NS
TNP-C57BL/6 antigen	20.2 ± 4.0	NS	29.5 ± 3.1	NS
Anti-TNP serum	18.2 ± 3.8	NS	33.3 ± 6.8	NS
TNP-BALB/c antigen + anti-TNP serum	49.5 ± 7.1	<0.01	39.2 ± 3.5	NS
TNP-C57BL/6 antigen + anti-TNP serum	16.3 ± 3.5	NS	66.8 ± 3.9	<0.001

Tumour allograft recipients were injected i.v. with cell membrane antigen derived from 10⁷ TNP-conjugated BALB/c or C57BL/6 cells with or without 0.1 ml rabbit anti-TNP-KLH serum i.p. Mice received 10⁷ tumour cells s.c. MTA, mean tumour area estimated on day 7. Values are for groups of 7–10 mice.

growth of an unmodified tumour allograft, the enhancement being specific and mediated by TNP-alloantigen/anti-TNP antibody complexes. Because the mice were injected only once with antigen and antibody it is perhaps not surprising that growth of the enhanced tumours was generally not progressive. The proposed mechanism of this enhancement has been outlined above. Although it is possible that more than one mechanism is operative in immunological enhancement, there is substantial evidence for the participation of antigen-reactive cell opsonization by antigen-antibody complexes⁴. We have, furthermore, found that TNP-modified H-2 antigens bind specifically to lymphocytes activated specifically in mixed lymphocyte culture and that ARCs to which complexes have been bound are taken up promptly by the reticuloendothelial system when injected i.v. into normal mice¹². The experiments reported here establish the direct immunosuppressive action of such complexes *in vivo*.

These findings may have special significance in two fields—in tumour immunology and in organ transplantation. Tumours often express neo-determinants that are either products of their own disturbed genome or of viral origin. The experiments making use of TNP-modified antigen provide an experimental model for the latter, the hapten taking the place of virally derived determinants. The ARCO phenomenon has already been illustrated in a syngeneic tumour system, using a spontaneous reticulum cell sarcoma (RCS) in SJL/J mice^{15,16}.

Table 3 Effect of TNP-BALB/c antigen on P815 tumour growth in A strain mice

Antigen dose (spleen cell equivalents)	MTA ± s.e. (mm ²)
None	24.6 ± 5.3
10 ⁸	23.7 ± 6.7
10 ⁷	23.8 ± 2.4
10 ⁶	29.7 ± 6.7
10 ⁵	23.1 ± 4.9

MTA, mean tumour area estimated on day 8. Values are for groups of six mice.

Although the viral aetiology of the RCS is uncertain it is possible that antibodies against viral products were involved.

As far as tissue and organ transplantation is concerned, preliminary data indicate that the survival of normal allografts, too, can be prolonged by the administration of TNP-modified donor antigen and anti-TNP antibody (I.V.H., unpublished). This approach may be indispensable if enhancement is to be attempted in clinical organ transplantation, for the preparation of the donor membrane antigen is quick (2½–3 h) and simple, and only a single antiserum directed against a well defined hapten such as TNP would be required to enhance grafts from any donor. Two further advantages would be that the antiserum can be prepared in animals or in monoclonal cell culture, thus eliminating the problem of having to prepare alloantisera in human volunteers; and that an anti-haptenic antibody, because it does not combine directly with the graft, cannot cause hyperacute rejection. Further studies on these lines are in progress.

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Specific suppressor cells in graft–host tolerance of HLA-identical marrow transplantation

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Graft-versus-host disease (GvHD) is a cell-mediated immunological reaction, and there is evidence in the rodent system that long-term survival without GvHD is associated with the presence of lymphocytes which specifically suppress the GvH reaction. We have accordingly developed an assay for the influence of circulating cells from patients with HLA-identical marrow grafts on the reaction of donor cells against host lymphocytes (collected before transplantation and modified by trinitrophenyl (TNP)). We find that patients without GvHD have radiation-sensitive circulating cells which specifically suppress the *in vitro* model of the GvH reaction. Our findings seem to have a bearing on graft–host tolerance and the causation of GvHD *in vivo*.

Most long-term patients after human marrow transplantation from HLA-identical siblings are healthy stable chimaeras who need no further immunosuppressive therapy^{1,2}. However, 30% of the chimaeras have mild to severe chronic GvHD characterized by lesions resembling those seen in autoimmune collagen vascular diseases^{3,4}, immunodeficiency⁵ and frequent bacterial infections⁶. We have described deposits of IgM and complement C'3 at the dermo-epidermal junction, raising the possibility that humoral anti-host immunity is involved in chronic GvHD⁷. The participation of cell-mediated immunity was suggested by the

Table 1 Suppressor cell assay

Culture	Responder cells	Stimulator cells* (irradiated)	'Suppressor' cells
Test	Donor	TNP-host	Chimaera†
Control A	Donor	TNP-host	Donor
Control B	Chimaera	TNP-host	Chimaera

Mononuclear leukocytes were separated in lymphocyte separation medium (Ficoll–Hypaque, Bionetics)^{8,10}. Host lymphocytes used as stimulating cells were cryopreserved before transplantation and thawed before use⁸. TNP-coupling with 10 mM trinitrobenzenesulphonic acid in phosphate-buffered saline at pH 7.4 for 10 min was done by published methods^{19–21}. Stimulator cells were treated with 1,600 rad irradiation. Responder and suppressor cells were prepared from freshly drawn blood. The cells were finally suspended in RPMI medium with 20% male AB or pooled human male serum, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. From each cell suspension, 0.05 ml containing 5 × 10⁴ lymphocytes was plated on to round-bottom plates (Linbro 76-013-05) as responder, stimulator or suppressor cells in ratios of 1:1:1. Cultures were set up in quadruplicate and incubated for 6 days at 37 °C in a humidified 5% CO₂ atmosphere. For the last 16 h of incubation, 2 µCi of ³H-thymidine (NEN) were added. The cultures were then collected and lymphocyte incorporation of the isotope-labelled thymidine was measured in a liquid scintillation counter.

$$\% \text{ Suppression} = 1 - \frac{\text{median c.p.m. of test cultures}}{0.5(\text{median c.p.m. of control cultures (A+B)})} \times 100$$

This formula has been described and justified¹⁰. A suppression of ≥30% was considered significant.

* Other stimulator cells were also used (see Tables 2,3).

† Chimaera cells = donor lymphocytes living in the marrow recipients.

Table 2 An example of results in patient 987

Responder	Suppressor	Stimulator			
		TNP-modified			
		Auto-logous*	Host	Donor	Un-related
Median c.p.m. of quadruplicate cultures					
Test					
Donor	Chimaera	327	6,320	11,352	7,602
		(5,912)*			
Controls					
A, Donor	Donor	1,830	9,573	9,509	11,677
			(4,931)†		14,649
B, Chimaera	Chimaera	208	1,227	2,593	4,683
Mean of A+B			5,401	6,051	8,180
% Suppression			94	–4	–39
			(–20)		19

A test was considered not evaluable when the MLC response of donor cells to a stimulator population was less than twice that of the autologous control. Test cultures consisted of 5 × 10⁴ donor lymphocytes as responders, 5 × 10⁴ stimulator cells and 5 × 10⁴ chimaera cells as suppressors. Control culture A consisted of 2 (5 × 10⁴) donor lymphocytes and 5 × 10⁴ stimulator cells. Control culture B consisted of 2 (5 × 10⁴) chimaera lymphocytes and 5 × 10⁴ stimulator cells. % Suppression was calculated according to the formula in Table 1 except for cultures indicated (*†). The high negative suppression which occurred less frequently than positive suppression resulted from an enhanced MLC response in the mixed culture. Chimaera cells alone often showed a lower MLC response than donor cells alone. The enhancement seen in mixed culture is probably due to an increased response of chimaera cells in the presence of donor cells. Chimaera cells may lack helper cells necessary for an adequate MLC response, and these are provided by the donor cells.

* In these cultures, the chimaera cells were irradiated with 1,600 rad.

† In these cultures, 5 × 10⁴ irradiated donor cells were added to the responder (donor) cells and stimulator cells. No control B was set up because irradiated suppressor cells gave no response to the stimulators.

observation of unidirectional reactivity of lymphocytes (of donor origin) from most chimaeras with chronic GvHD in response to cryopreserved host lymphocytes in mixed leukocyte culture (MLC)^{8,9}. It was tempting to speculate that this finding represented the *in vitro* equivalent of a continuing immunological reaction which manifested itself *in vivo* as chronic GvHD. Conversely, chimaeras without GvHD lacked *in vivo* evidence for immunological reactions, and their lymphocytes in general did not display MLC reactivity to host cells⁸. We have also reported the presence in patients with chronic GvHD of circulating cells that suppressed the ability of lymphocytes from the marrow donor to proliferate in response to unrelated lymphocytes and/or to concanavalin A^{10,11}. Others described abnormal

Table 3 Suppressor cell activities in long-term patients

Patients	UPN	Underlying disease	Test day post-grafting	Stimulator cells			
				TNP-modified			Unrelated
				Host	Donor	Unrelated	
				% Suppression			
Without GvHD (<i>n</i> = 14)	656	AML	732	36	ND	20	-3 [†]
			1,460	55	ND	ND	-6
	674	AML	1,012	47	21	7	20
	679	ALL	764	50	ND	NR	-29
			923	38	-12	3	10
			1,066	33	ND	-18	-38
	732	AA	349	45	ND	-24	-23
	737	AA	831	66	86	-65	7
	741	AA	842	57	-100	78	17
	771	AA	996	64	NR	-23	-50
	776	AML	371	34	ND	0	9 [†]
	777	AA	822	56	23	-36	22
	896	AA	364	79	75	-24	-41
	959	AML	381	71	ND	14	-59
	987	ALL	362	94	-4	-39	19
	1,019	AML	331	-51	ND	ND	ND
	1,082	AML	374	36	NR	-18	-4
Chronic GvHD (<i>n</i> = 23)	612	AA	1,080	-100	-71	27	-5
	630	AA	911	27	25	18	12
	644	ALL	959	-68	-12	-15	30
	705	AML	745	-34	-55	NA	83
	723*	AMML	868	-27	3	11	-4
	734	AA	830	53	-35	11	36
	800	CML	652	1	53	23	42
	804	AMML	699	-22	ND	-23	-10
	838	AA	734	-29	ND	59	30
	886	ALL	350	69	16	NA	22
	889	AMML	372	48	-23	0	-15
	909	AML	535	10	70	-100	NA
	911	ALL	368	1	38	4	-53
	957	CML	354	-15	NR	-25	62
	962	AML	336	57	68	44	-21
	993	AML	394	-57	14	-9	-37
	995	AML	364	-88	-100	NA	78
	1,002*	CML	357	-100	NR	NR	2
	1,020*	AMML	390	21	ND	ND	-95
	1,023	AML	211	84	NR	11	35
	1,068	AMML	238	-30	ND	60	-98
	1,083	Hodgkin's lymphoma	171	-44	NR	NR	74
	1,233	AMML	131	1	ND	ND	-19

UPN, unique patient number. Underlying disease: AA, aplastic anaemia; ALL, acute lymphoblastic leukaemia; AML, acute myelogenous leukaemia; AMML, acute myelomonocytic leukaemia; CML, chronic myelogenous leukaemia. Suppression of $\geq 30\%$ is indicated by bold figures. ND, not done; NA, not applicable, when chimaera MLC response (control B) > donor MLC response (control A)¹⁰; NR, no response, when donor MLC response to the stimulators (control A) < 2 × that of the autologous control.

† Results previously reported¹⁰.

* These patients had extremely limited chronic GvHD.

numbers of TH₂ cells in some patients with GvHD¹². Studies of *in vitro* immunoglobulin synthesis showed a lack of helper cells and the presence of suppressor cells in chimaeras with chronic GvHD¹³. Such cellular imbalance may explain the severe immunodeficiency seen in patients with chronic GvHD. Stable long-term chimaeras generally did not show evidence for similar immunological deficiencies.

The mechanisms maintaining stable graft-host tolerance in healthy long-term survivors after marrow transplantation have been recently investigated. A role for serum-blocking factors has been ruled out in both canine and human recipients of marrow grafts^{14,15}. Although the involvement of specific suppressor cells in the maintenance of transplantation tolerance has been established by many investigators¹⁶, in clinical marrow transplantation, such evidence is lacking due to the difficulty of studying a model with HLA identity. In the animal models, we have presented circumstantial evidence for an active suppressor mechanism in stable canine radiation chimaeras¹⁷, and Tutschka *et al.* suggested that the development of specific and nonspecific suppressor cells in rat chimaeras after the resolution of GvHD accounted for the establishment of specific graft-host tolerance¹⁸.

In the present study, the *in vitro* model was devised to test

chimaera cells for their ability to suppress the response of donor lymphocytes to TNP-modified host cells stored before transplantation. TNP was used because donor lymphocytes did not respond to unmodified host cells in the MLC due to HLA identity. The TNP moiety of trinitrobenzene sulphonate covalently links to ϵ -amino groups of the lysines of cell-surface proteins. Studies in mice have shown that cytotoxic cells generated *in vitro* after sensitization with TNP-modified autologous cells recognize not only the TNP moiety but also self components coded for by genes mapping in H-2 (ref. 19). Studies on the human cytotoxic response to TNP-modified cells have suggested that almost half of the cytotoxicity generated was directed against polymorphic determinants thought to be HLA linked²⁰. Some of the cytotoxic activity seemed to be directed against TNP in association with relatively nonpolymorphic antigens widely shared among humans²¹. These findings were interpreted as evidence for considerable diversity of the types of cell-surface determinants that human cytotoxic cells can recognize in association with TNP.

The hypothesis underlying the present study was that polymorphic determinants not linked with HLA can be recognized in association with TNP in a primary MLC assay and that these determinants have a role in human GvHD. We presumed that

suppression of the MLC reactivity by lymphocytes from the chimaeras without GvHD would be specific for recognition of TNP-associated host determinants but would not impair recognition of TNP-associated determinants shared between the siblings or with unrelated individuals. Conversely, recognition of TNP-associated determinants by donor lymphocytes would not be abrogated by lymphocytes from chimaeras with chronic GvHD.

Thirty-seven patients with aplastic anaemia, leukaemia or Hodgkin's lymphoma (Table 3), 8–50 yr old, were treated by high-dose cyclophosphamide, either alone or in combination with 1,000 rad total body irradiation, in preparation for a marrow graft from a genotypically HLA-identical sibling^{1,2}. All were given immunosuppression with intermittent methotrexate for no longer than 3 months post-grafting. Fourteen were healthy chimaeras without GvHD and 23 had minimal to severe chronic GvHD. Tests were carried out between 131 and 1,460 days post-grafting. At the time of testing six patients were under immunosuppressive treatment for chronic GvHD⁴ while the remainder were untreated.

Details of the lymphocyte separation, TNP modification and the assay system are described in Table 1 legend. Lymphocytes from 37 marrow donors were stimulated by TNP-modified lymphocytes from their recipients collected before transplantation (TNP-modified host cells) with stimulation indices (SI) ranging over 2–15.8 (mean 4.3). Mononuclear cells from the peripheral blood of the chimaeras were tested for their ability to suppress the reactivity of the donor lymphocytes to TNP-modified host lymphocytes. In addition, we tested the effect of chimaera lymphocytes on the response of the donor lymphocytes to unmodified, unrelated stimulating cells from healthy individuals ('nonspecific' suppression)¹⁰. To test for specificity, the effect of chimaera lymphocytes on the responses of the donor lymphocytes to TNP-modified donor and TNP-modified unrelated lymphocytes was also studied.

Table 2 shows an example of the results in a patient without GvHD (UPN 987) studied 362 days after transplantation. The circulating lymphocytes from the chimaera, when mixed with donor lymphocytes in an MLC, suppressed the response to TNP-modified host cells by 94%. The suppressor activity was abrogated by exposure of chimaera cells to 1,600 rad irradiation. (Radiosensitivity of the suppressor cells was also shown in four out of four other cases studied.) The chimaera's lymphocytes did not suppress the response of donor lymphocytes to TNP-modified donor nor unrelated TNP-modified or unmodified cells.

Table 3 summarizes the results in the 37 patients studied. Lymphocytes from 13 of 14 healthy chimaeras suppressed the response of donor lymphocytes to TNP-modified host lymphocytes compared with those of only 5 of 23 chimaeras with chronic GvHD ($P < 0.0005$, χ^2 test). Lymphocytes from none of the healthy chimaeras suppressed the ability of their marrow donor lymphocytes to respond to unmodified unrelated lymphocytes compared with those of 9 of the 22 chimaeras with chronic GvHD tested ($P < 0.01$). We were able to test two patients (UPNs 656 and 679) from the former group repeatedly and show comparable results. Suppression of donor lymphocyte responses to TNP-modified self and TNP-modified unrelated lymphocytes was seen in 2/7 and 1/14 cases among healthy chimaeras and in 4/14 and 3/16 cases among chimaeras with chronic GvHD (differences not significant).

The results of the study are in agreement with our hypothesis. Most patients without GvHD had circulating cells 'specifically' suppressing donor lymphocyte reactivity to TNP-modified host cells while most patients with chronic GvHD lacked specific suppressor cells. This suggests that stable graft-host tolerance after human marrow grafting is maintained by specific suppressor cells. Conversely, the lack of specific suppressor cells in patients with chronic GvHD allows an immunological reaction to occur which manifests itself *in vivo* as GvHD and *in vitro* as unidirectional reactivity of chimaera lymphocytes (that is, donor lymphocytes in the patient) against cryopreserved host

lymphocytes in MLC⁸. The nature of these suppressor cells and the time of their appearance in the blood after transplantation are unknown.

The present study also confirms a previous observation^{10,11} on the existence of another class of suppressor cells, present in patients with and absent in those without chronic GvHD, which nonspecifically suppresses the response of marrow donor lymphocytes to unmodified unrelated lymphocytes in MLC. These nonspecific suppressor cells may be one explanation for the severe immunodeficiency⁵ and the recurrent infectious complications⁶ characteristic of patients with chronic GvHD.

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Human Ia molecules carrying DC1 determinants differ in both α - and β -subunits from Ia molecules carrying DR determinants

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Several genetic markers of human Ia molecules, each recognized by specific alloantisera, have been defined: DR 1,2,3,4,5,w6,7,w8,w9,w10. They are controlled by a single locus, HLA-DR. In addition alloantigenic specificities have been identified which are associated, at the population level, with two or more of the above DR specificities. They have been called 'supertypic specificities'. DC1 supertypic specificity (nomenclature equivalents: MB1, MT1, LB12) has attracted particular attention, because although exhibiting a population association with DR1, 2 and w6, DC1 is carried by a different molecular species^{1–3}. Possibly, DC1 may represent the first genetic marker of a locus different from the DR locus but in strong linkage disequilibrium and therefore probably closely linked to it. Here we report the development of a monoclonal reagent specifically directed against DC1 and its utilization in a structural analysis of DC1 molecules as compared with Ia molecules carrying different DR determinants.

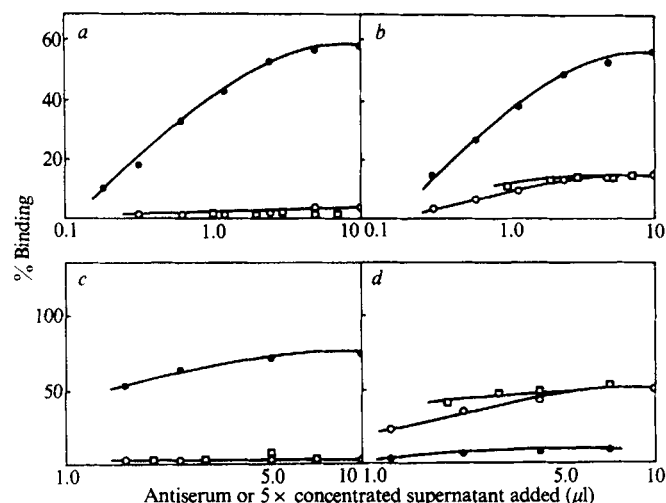


Fig. 1 A detergent-solubilized Ia preparation from the Daudi cell line was ^{125}I -labelled by the chloramine T method. Limited papain digestion was performed at 1:40 enzyme to protein ratio (bovine serum albumin as carrier protein) for 30 min at 37°C. Papain-unsplit and 'papain-split' fractions were separated on a column of Biogel A 1.5 and further purified by LcH affinity chromatography. Details about the whole procedure have been previously reported⁸. Tests of the two fractions with the different antisera were performed by the double precipitation direct binding test¹. Aliquots of antigen containing 15,000 c.p.m. were incubated with different antiserum dilutions for 16 h at 20°C in 0.075 M Tris-HCl buffer, pH 7.8, containing 0.2% Renex-20 detergent and 0.02% bovine serum albumin. Precipitating antiserum (either sheep anti-human or rabbit anti-mouse immunoglobulin) was then added at equivalence and the radioactivity in the washed precipitate determined. Per cent binding was expressed relative to the binding of the same antigenic preparation by a rabbit antihuman Ia antiserum. Further details are given elsewhere². Absorption of the papain-split fraction was achieved by reacting 200,000 c.p.m. of antigen with 20 μl of either Fe131/4 or Fe88/37 alloantiserum for 16 h at 20°C. The supernatant recovered after precipitation with sheep anti-human immunoglobulin was then further tested with the different antisera at varying dilutions. $\circ$, Fe131/4; $\bullet$, Fe88/37; $\square$, BT3/4. *a*, Daudi unsplit; *b*, Daudi split (not absorbed); *c*, Daudi split (absorbed with Fe 131/4, anti-DC1); *d*, Daudi split (absorbed with Fe 88/37, anti-DRw6).

The monoclonal antibody BT3/4 is secreted by a hybridoma obtained by the method described by Geffer *et al.*⁴. Briefly, BALB/c mice were immunized intraperitoneally at 10-day intervals with 10^7 E-rosetting cells obtained from a secondary mixed lymphocyte culture (MLC). A mouse was killed 4 days after the third injection and its spleen was fused with P3/X63-Ag8-U1 myeloma cells. Fourteen days after fusion the supernatant of each cell was assayed by indirect immunofluorescence on activated T cells. Positive cultures were cloned on soft agar essentially as described by Coffino *et al.*⁵, and the clone BT3/4 passed into ascites form for mass production of monoclonal IgG1. When tested on a panel of cell lines and peripheral blood lymphocytes by indirect immunofluorescence, BT3/4 invariably reacted only with lymphoblastoid B-cell lines and B lymphocytes from donors with a DR phenotype including DR 1, 2 or w6 (Table 1). The specificity of BT3/4 is similar to that previously reported^{6,7} for the monoclonal antibody Genox 353.

At the molecular level, the specificity of BT3/4 was tested on purified Ia preparations from the Daudi cell line (Drw6⁺, DC1⁺). As reported previously, after limited papain digestion, two fractions of Daudi Ia can be separated by gel filtration: 'papain-unsplit' fraction, retaining the approximate size of untreated molecules, and a 'papain-split' fraction, with decreased molecular weight. DC1 molecules were present in increased proportion in the 'papain-split' fraction⁸. This provided a convenient test of the specificity of the monoclonal BT3/4, which, as shown in Fig. 1 (*a* and *b*) did not react with the 'papain unsplit' fraction, while reacting similarly to the reference anti-DC1 alloantiserum Fe131/4 with the 'papain-split' fraction. Moreover, the reactivity of BT3/4 was completely abolished by absorption with Fe 131/4, whereas absorption with Fe 88/37 (anti-DRw6) induced a parallel increase of binding of Fe 131/4 and of monoclonal BT3/4 (Fig. 1, *c* and *d*). This shows that the molecules which carry the DC1 determinant as recognized by a specific anti-DC1 alloantiserum are the same molecules which carry the determinant recognized by the BT3/4 monoclonal antibody.

DC1 antigen was purified by immunoabsorption to Sepharose-bound BT3/4 from the lymphoblastoid cell lines

Table 1 Reactivity of monoclonal BT3/4 with lymphoblastoid B-cell lines and peripheral blood B cells

DR phenotype		Reactivity with BT3/4
Cell lines		
LG2	1, 1	+
WT18	2, 2	+
LG17	2, 2	+
LG38	5, 5	-
WT48	5, 5	-
WT52	w6, w6	+
LG28	w6, w6	+
Daudi	w6, -	+
Raji	3, w6	+
M. W.	2, 4	+
Donors		
A. B	2, w6	+
G. D.	4, 5	-
M. F.	1, 4	+
F. C.	5, 7	-
A. M.	5, -	-
R. A.	2, 4	+

Lymphoblastoid B-cell lines LG2, LG17, LG28 were provided by Dr W. Leibold and were derived as previously described²⁷. All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Seromed). B cells were purified from peripheral blood by already described techniques²⁸. Indirect immunofluorescence was carried out as previously described²⁸.



Fig. 2 Autoradiograph of a 12.5% SDS-PAGE of unreduced DC1 and DR antigens. 10^7 WT52 cells were lysed with 0.5% Nonidet P40 and centrifuged to remove nuclei and cell debris. DC1 and DR molecules were purified from the lysate by the immunoabsorption method described in detail in ref. 9. BT3/4 and PTF29 were coupled to Sepharose 4-B by the cyanogen bromide technique. A Sepharose-bound monoclonal antihuman IgE was used as control. The antigens were eluted with 5% SDS and labelled using the chloramine T method according to Greenwood *et al.*²⁹. SDS-PAGE was carried out in slab gels using the discontinuous Tris buffer system as previously reported⁹. Non-reducing conditions were used to increase separation of α - and β -chains.

LG2 and WT52, carrying DR allospecificities 1 and w6, respectively. After iodination, the bound material was analysed in SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2).

DC1 antigen is composed of two chains with an apparent molecular weight very similar to that of DR α - and β -chains. However, unreduced DC1 β -chain moves slightly faster than DR β -chain at an apparent molecular weight of 23,000.

The structure of DC1 antigens isolated from the two lines was further compared by two-dimensional peptide mapping with that of DR 2, 5, w6 and 7 similarly purified with the monoclonal PTF 29.12 (ref. 9). Figure 3 shows the comparison between peptic digests obtained from α chains of the two DC1 preparations and of the four DR antigens; DC1 α -chains isolated from DR 1 and DR w6 cell lines are identical, but bear no resemblance to any of the four DR α -chains. In Fig. 4 the equivalent comparison is reported for DC1 and DR β -chains. As previously reported⁹, the maps of the β -chains from the four different DR alleles show, besides the expected degree of alleotypic variability, a core of 12 invariant peptides; none of these peptides is present in the DC1 β -chain. Again β -chains of DC1 purified from the DR 1 and DR 6 cell lines are identical.

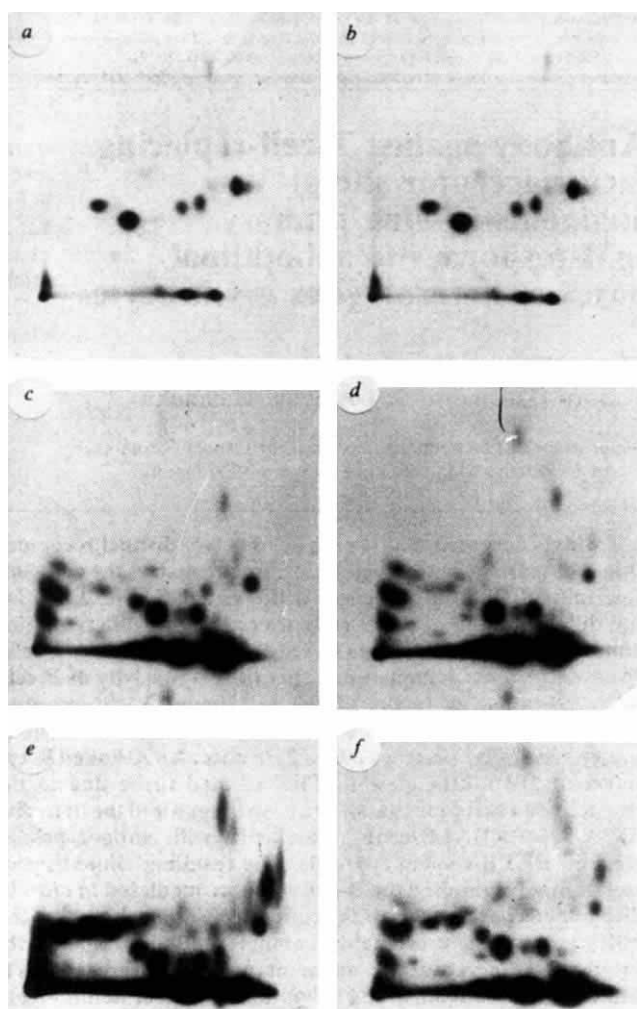


Fig. 3 Autoradiographs of peptide maps of ^{125}I -labelled α -chains of DC1 and DR antigens. α - and β -chains were eluted from the gel and processed for peptide mapping as previously described⁹. Briefly, after complete reduction with dithiothreitol and alkylation with iodoacetamide, α - and β -chains were digested with pepsin (1:50 enzyme to protein ratio, 18 h at 37°C in 100 μl of formic acid/acetic acid/water (1:4:45)). Two-dimensional peptide maps were obtained by a technique⁹ based on the method of Feinstein³⁰. a, DC1 α -chain from LG2 (DR1, 1); b, DC1 α -chain from WT52 (DRw6, W6); c, DR α -chain from WT52; d, DR α -chain from IBW9 (DR7, 7); e, DR α -chain from LG17 (DR2, 2); f, DR α -chain from WT48 (DR5, 5).

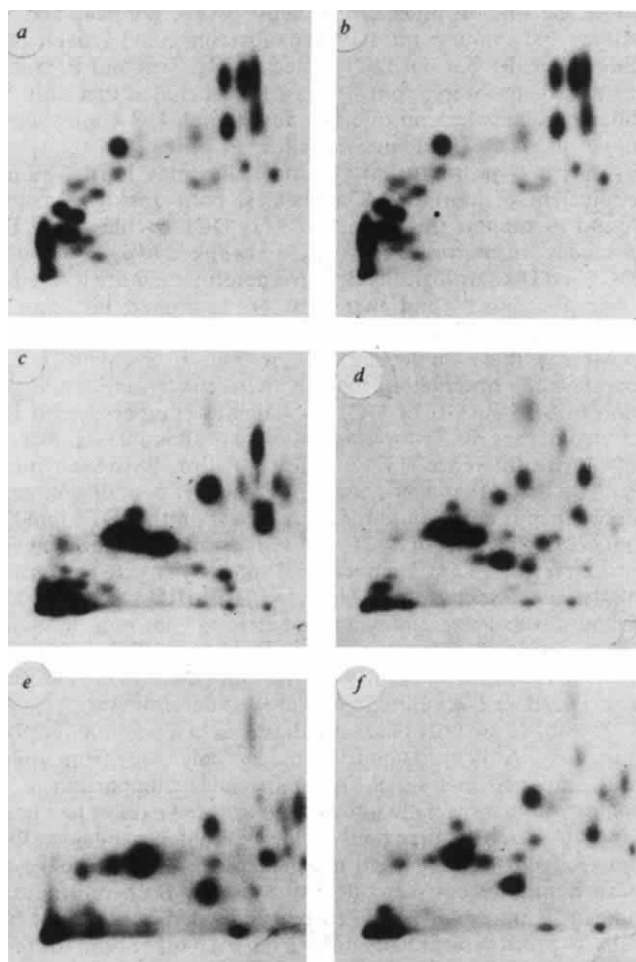


Fig. 4 Autoradiographs of peptide maps of ^{125}I -labelled β chains of DC1 and DR antigens. a, DC1 β -chain from LG2 (DR1, 1); b, DC1 β -chain from WT52 (DRw6, w6); c, DR β -chain from WT52; d, β -chain from IBW9 (DR7, 7); e, DR β -chain from LG17 (DR2, 2); f, DR β -chain from WT48 (DR, 5). The detailed comparison of DR chains from different alleles is given elsewhere⁹. DR peptide maps similar to those shown in ref. 9 have been included here to facilitate comparison with the peptide maps of DC1 chains.

It should be stressed that this type of technique, involving comparison of molecules labelled in a single amino acid, underestimates the degree of homology of their primary sequences⁹. In fact, a single amino acid substitution is sufficient to cause a peptide to move to a different position. However, it can be safely concluded that DR and DC1 differ both in their α - and β -chains and that the minimum number of different amino acids corresponds to the number of spots present in the maps (α and β respectively). A precise quantitation must await sequence data.

The data presented are better discussed in their possible analogies to the murine Ia system. Murine Ia includes at least two subsets of molecules with similar basic structures: I-A molecules and I-E molecules. Based on recent data^{10,11}, the A α - and A β -subunits as well as the E β -subunit are controlled by genes in the I-A subregion, whereas the E α -subunit maps in the I-E subregion. Polymorphic structural differences can be detected mainly on the three I-A subregion-controlled subunits. Several structural and serological findings, including amino acid sequence data, indicate homology between murine I-E molecules and 'human Ia molecules'^{12,13}. These data were obtained before the recognition of separate subsets of Ia molecules probably controlled by different loci^{1,14,15}. Therefore it is very likely that the major molecular subset, that is, the DR locus-controlled molecules, were actually tested. In fact, DC1 molecules constitute a minor fraction as compared with DR molecules⁸ and their presence would not have affected the

sequence analysis much above 'noise level'. We have actually shown that a mouse anti I-E^k alloantiserum could actively bind DR molecules but not DC1 molecules (R. Tosi and P. Ivanyi, unpublished observation). It must be concluded that only the homology between murine I-E and human DR-controlled Ia molecules is clearly demonstrated.

Thus it is possible that DC molecules may be the human equivalent of murine I-A molecules. Four analogies can be found to support this hypothesis: (1) DC1 β -chains and DR β -chains are controlled by very closely linked loci. In fact, both DC1 and DR serologically detected determinants are located on their β -chains^{1,15} and there are no confirmed instances of DC/DR recombination. (2) A β -chains have been shown to possess a lower molecular weight than E β -chains¹⁶. This parallels the observation reported here concerning the migration in SDS-PAGE of DC1 β -chains as compared with DR β -chains. (See ref. 15 for a discussion of the possible significance of this size difference.) (3) Extensive variations have been found, both by limited sequence analysis^{12,17} and by peptide analysis¹⁸ between murine A α and E α . Our data show that DC1 and DR molecules differ not only, as expected, in their β -chains, but also in their α -chains. (4) A monoclonal antibody directed against an allotypic specificity of rat Ia molecules, MRC OX3, was also found to recognize a polymorphic determinant both in mouse and in man^{19,20}. In man, it reacted only with DR 1, 2 or w6 positive cell lines, thus behaving as an anti-DC1. In the mouse, it recognized an I-A subregion-controlled determinant.

The above are only indirect indications of a possible DC/I-A homology. A direct demonstration can only come from amino acid sequence analysis of DC1 molecules. Comparison of α -chains will be especially informative as these exhibit less polymorphic variations than do β -chains¹². Whatever the homology between single human and murine Ia loci, the data suggest a basic similarity between the two systems. Both murine and human Ia molecular pools can be subdivided into at least two subsets which substantially differ from each other both for their α - and β -chains. A similar conclusion has been reached for rat Ia antigens²¹. The association of DC1 β -chains with a distinctive species of α -subunit definitely rules out the possibility that the DC1 supertypic specificity is controlled by the HLA-DR locus. In fact, the different DR allelic products have been shown to possess marked structural variations in their β -subunits but not in their α -subunits^{9,22,23}, while the structure of the α -subunit associated to the DC1 molecule has clearly distinctive features. This confirms previous immunochemical and genetic data which assigned DC1 to a separate, non-DR, locus.

An interesting point, as in the mouse system, is the non-interchangeability of α -chains in their association with β -chains encoded by different loci. This is probably due to a selective post translational association, as coordinated translation of α - and β -chains from the same messenger seems unlikely because separate messenger RNAs for the two chains can be isolated²⁴, and because A β -chains can associate with A α -chains transmitted by the homologous chromosome²⁵. Markert and Creswell²⁶ have recently reported the existence of at least two species of β -subunits and at least three species of non-interchangeably associated α -subunits. Apart from the interpretation given by the authors on the localization of alloantigenic determinants on the different chains, the above data, in agreement with ours, also imply the existence in man of Ia molecular subsets differing for both α - and β -chains and carrying alloantigenic determinants which are transmitted in strong linkage disequilibrium with DR determinants.

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Antibody against T cell-replacing factor acceptor site(s) augments *in vivo* primary IgM responses to suboptimal doses of heterologous erythrocytes

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It is widely accepted that B cells possess two distinct receptors. One is the surface immunoglobulin that recognizes the antigenic determinant; the other transmits the signals for proliferation and differentiation of the B cells after receiving differentiation stimuli mediated by a soluble factor derived from helper T cells. Previously¹⁻⁴, we demonstrated that the responsivity of B cells to T cell-replacing factor (TRF) was under X-chromosome control, as determined by genetic analysis of the TRF-nonresponsiveness of B cells from DBA/2Ha mice. An X-linked B-cell defect of DBA/2Ha mice to TRF seemed to be due to the absence of an acceptor site(s). We have immunized the defective (DBA/2Ha $\times$ BALB/c δ)F₁ male mice with antigen-primed parental BALB/c splenic B cells. The resulting alloantiserum preferentially inhibited the B-cell response mediated *in vitro* by TRF added continuously to the culture. We report here that this antiserum, which presumably contains antibody against the putative TRF-acceptor site, augments primary IgM responses to simultaneously administered suboptimal doses of heterologous erythrocytes injected into TRF high-responder animals. These augmented IgM-PFC (plateau-forming cell) responses were also observed in other X-linked B-cell defective CBA/N mice, but not in TRF low-responder DBA/2Ha mice, suggesting that the putative TRF-acceptor site(s) functionally detectable by this antiserum is distinct from the Lyb 3 and Lyb 5 components.

To prepare alloantiserum containing anti-TRF-acceptor site(s) antibody, (DBA/2Ha $\times$ BALB/c)(DC)F₁ male mice (TRF-low responder animals) were immunized intraperitoneally (i.p.) with splenic B cells (2×10^7) from DNP (dinitrophenyl)-KLH (keyhole limpet haemocyanin)-primed BALB/c mice of a high TRF-responder parental strain, with

Table 1 Enhancing activity of (DC)F₁ male anti-BALB/c B-cell antiserum on PFC responses in BALB/c and BALB/c *nu/nu* mice stimulated with graded doses of SRBC

Strain	Immunization (no. SRBC × 10 ⁶)	Serum	Direct PFCs per spleen	Direct PFCs per 10 ⁶ spleen cells
BALB/c	0.1	Control	337 (1.63)	2.42 (1.50)
		(DC)F ₁ (♂)anti-BALB/c	4,808 (1.38)	24.1 (1.50)
	1.0	Control	1,682 (1.47)	12.1 (1.49)
		(DC)F ₁ (♂)anti-BALB/c	6,832 (1.40)	53.0 (1.04)
	10.0	Control	29,337 (1.23)	190.0 (1.19)
		(DC)F ₁ (♂)anti-BALB/c	48,245 (1.07)	290.0 (1.09)
BALB/c <i>nu/nu</i>	1.0	Control	477 (1.20)	2.17 (1.13)
		(DC)F ₁ (♂)anti-BALB/c	4,604 (1.19)	21.5 (1.25)
	10.0	Control	3,342 (1.06)	49.2 (1.14)
		(DC)F ₁ (♂)anti-BALB/c	9,997 (1.04)	113.3 (1.25)

BALB/c or BALB/c *nu/nu* mice, aged 5–7 weeks, were immunized i.v. with various amounts of SRBC in combination with (DC)F₁ male anti-BALB/c antiserum (1:40) or control serum, in a volume of 0.2 ml. PFC responses 4 days after antigen stimulation and antiserum injection are shown. Results are expressed as geometric means ± s.e.m.

4 mg aluminium hydroxide gel and *Bordetella pertussis* vaccine (1 × 10⁹), followed by i.p. injection of DNP-primed B cells without adjuvant once a week for 5 weeks. Seven days after the final immunization, each mouse was partially bled once a week for 4 weeks and the serum pooled. The activity of the antiserum was determined by measuring the inhibition of TRF-mediated antibody response according to methods used elsewhere.

To determine whether the reaction of the TRF acceptor on the surface of the B cells with anti-TRF acceptor antibody altered the immune reactivity of these cells, we examined the PFC responses of BALB/c mice on stimulation with 1 × 10⁶ sheep red blood cells (SRBC) alone or in combination with graded doses of anti-TRF acceptor site antiserum or control serum. As shown in Fig. 1, mice stimulated with SRBC alone or in combination with control serum produced ~500–1,500 PFCs per spleen (5–10 PFCs per 10⁶ cells), whereas mice injected with SRBC and an optimal concentration of anti-TRF acceptor site antiserum produced a 5- to 10-fold increase in anti-SRBC PFCs. Studies of the time course of this enhancing effect of the antiserum revealed that the peak response occurred on day 4 after antiserum injection in combination with a suboptimal dose of SRBC, and that by day 8 this response had declined to control serum levels (data not shown). Mice injected with (DC)F₁ male anti-BALB/c B-cell antiserum alone without antigen also produced much fewer (1.5–1.8 times the background) but significant PFC responses to SRBC. This increase in PFCs due to the antiserum was observed in anti-HRBC (horse red blood cell) plaques as well (data not shown). These findings suggest that the anti-TRF acceptor antiserum acts synergistically with antigen to produce enhanced antigen-specific PFC responses. However, this does not exclude the possibility that anti-TRF-acceptor antibody alone induces polyclonal B-cell activation.

To demonstrate that the enhancing effect of (DC)F₁ male anti-BALB/c B-cell antiserum on the PFC response of BALB/c mice is particularly evident when the triggering signals provided by both antigen and helper T cells are relatively low, we examined the effect of anti-TRF-acceptor antiserum: (1) in the presence of decreased concentrations of antigen, and (2) in the absence of T cells. Addition of antiserum (Table 1) markedly enhanced the PFC response to low doses of SRBC from 1 × 10⁵ to 1 × 10⁶. In contrast, the enhancing effect of antiserum on PFC responses of those mice stimulated with large doses of SRBC was marginal. In a further experiment, the ability of (DC)F₁ male anti-BALB/c B-cell antiserum to substitute for helper T-cell activity in the induction of a primary PFC response was tested in *nu/nu* mice. BALB/c *nu/nu* mice produced a low number of anti-SRBC PFCs after priming with 1 × 10⁶ SRBC plus control serum, compared with the IgM anti-SRBC PFC response of BALB/c mice in the same experimental conditions. However, the administration of (DC)F₁ male anti-BALB/c B-cell antiserum with SRBC to these *nu/nu* mice resulted in a striking increase in anti-SRBC PFC responses (Table 1).

It has been reported that CBA/N mice lack a subpopulation of B cells that appears late in ontogeny^{5–7} and responds to

thymus-independent type 2 antigens, such as Ficoll and dextran^{8,9}. As the defect is X-linked and recessive, an alloantiserum denoted anti-Lyb 3 that would react exclusively with this subset of B cells in all mouse strains except CBA/N was raised by immunizing (CBA/N × BALB/c)F₁ male mice with BALB/c spleen cells⁵. Huber *et al.*⁵ demonstrated that this anti-Lyb 3 antiserum acts synergistically with antigen to produce enhanced antigen-specific PFC responses in various strains of mice except CBA/N. We decided to test whether this augmenting effect of (DC)F₁ male anti-BALB/c B-cell antiserum is due to an interaction between antibody and TRF-acceptor sites on the surface of B cells, and also to test whether or not the TRF-acceptor sites detected by this antibody are identical to or closely associated with the Lyb 3 molecules. We examined the effect of (DC)F₁ male anti-BALB/c B-cell antiserum on primary anti-SRBC

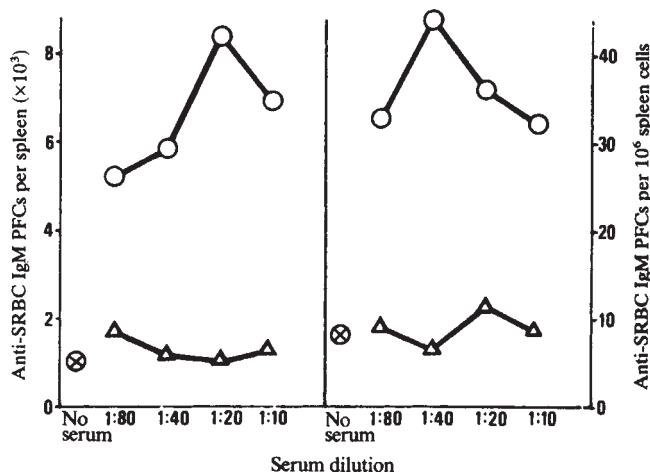


Fig. 1 Dose-related augmenting effect of (DC)F₁ male anti-BALB/c B-cell antiserum on anti-SRBC PFC responses. (DBA/2Ha × BALB/c)(DC)F₁ male anti-BALB/c B-cell alloantiserum containing anti-TRF-acceptor site antibody was prepared by the method described elsewhere⁷. Briefly, after treatment with monoclonal anti-Thy 1.2 antiserum and complement, splenic B cells from DNP-KLH-primed B cells of mice of a high TRF-responder strain (BALB/c) were used as immunizing cells. Low TRF-responder (DC)F₁ male mice were injected i.p. with these DNP-primed B cells (2 × 10⁷), with 4 mg aluminium hydroxide gel and *B. pertussis* vaccine (1 × 10⁷), followed by i.p. injections of DNP-primed B cells without adjuvant once a week for 6 weeks. Seven days after the final immunization, partial bleeding was performed on each mouse once a week for 4 weeks and the serum pooled. Six-week old BALB/c mice were immunized i.v. with 1 × 10⁶ SRBC alone (⊙) or in combination with varying dilutions of (DC)F₁ male anti-BALB/c B-cell antiserum (○) or normal (DC)F₁ mouse serum (Δ) as a control, in a volume of 0.2 ml. After 4 days the mice were killed and the number of IgM plaque-forming cells (PFC) to SRBC was determined per spleen and per 10⁶ spleen cells. Each point represents the mean PFC response of six mice from a representative experiment of a series of five.

Table 2 The effect of (DC) F_1 male anti-BALB/c B-cell antiserum on various strains of mice

Strain	Serum	Direct PFCs per spleen	Direct PFCs per 10^6 spleen cells
BALB/c	Control	518 (1.09)	4.31 (1.17)
	(DC) F_1 (δ)anti-BALB/c	7,590 (1.23)	42.5 (1.24)
C57BL/6	Control	649 (1.28)	7.10 (1.27)
	(DC) F_1 (δ)anti-BALB/c	3,934 (1.09)	39.0 (1.02)
DBA/2Ha	Control	104 (1.44)	1.48 (1.22)
	(DC) F_1 (δ)anti-BALB/c	326 (1.33)	1.78 (1.29)
CBA/N	Control	165 (1.37)	3.30 (1.44)
	(DC) F_1 (δ)anti-BALB/c	3,030 (1.03)	73.6 (1.12)

Various strains of mice, aged 6–8 weeks, were immunized i.v. with 1×10^6 SRBC in combination with (DC) F_1 male anti-BALB/c antiserum (1:40) or control serum, in a volume of 0.2 ml. Results are expressed as geometric means $\pm$ s.e.m.

responses in various strains of mice. As shown in Table 2, enhancement of anti-SRBC responses was observed not only in BALB/c but also in C57BL/6 and CBA/N mice. In contrast, intravenous (i.v.) injections of varying doses of anti-TRF acceptor serum failed to augment the anti-SRBC PFC response in DBA/2Ha mice, indicating that the augmenting effect was clearly due to a specific reaction of this antiserum with a component which is absent from the DBA/2Ha mice (Table 2). The fact that injection of CBA/N mice with SRBC in combination with antiserum produced more than a 10-fold increase in anti-SRBC PFCs indicated that enhancement of the anti-SRBC responses in CBA/N mice is clearly due to a specific reaction of this serum with a component that is different from Lyb 3 and Lyb 5, which are absent from CBA/N mice.

Our results clearly demonstrate that passive administration of anti-TRF acceptor site antiserum in combination with suboptimal doses of SRBC in TRF high-response animals can induce augmented primary anti-SRBC IgM responses (Fig. 1 and Table 2), and that stimulation of the acceptor site(s) on antigen-reactive B cells with antibody allows more efficient triggering of B cells by antigen. The ability of anti-TRF acceptor site antibody to substitute for helper T-cell activity in the induction of a primary response is obvious from the fact that anti-TRF acceptor site antiserum administered to *nu/nu* mice resulted in the production of a significant PFC response (Table 1). Although the positive response of CBA/N mice to (DC) F_1 male anti-BALB/c B-cell antiserum supports the notion that the TRF-acceptor site(s) which we detected here is different from Lyb 3 (and -5 and -7) components, another possibility is that there are several (at least more than one) kinds of TRF-acceptor site molecules on B cells, especially in the light of reports by Parker *et al.*¹⁰ and Ahmed *et al.*¹¹ on the inability of unprimed B cells from CBA/N mice to respond to TRF (concanavalin A (Con A) supernatant). As anti-Lyb 3 antiserum was also able to augment a primary anti-SRBC response *in vivo* to suboptimal doses of antigen, and thus showed the same functional characteristics as our antiserum, Lyb 3 could be another acceptor molecule for the TRF originated from Con A supernatant. The interrelation between the TRF acceptor site(s) detected here and its Lyb 3 components awaits further investigation.

Note that there is a discrepancy between the triggering effect of anti-TRF acceptor antibody as described here and the inhibitory effect of the same antibody on B cell-triggering mediated by TRF as described previously^{2–4}. Recently, we found that antigen-primed B cells could be triggered *in vitro* by anti-TRF-acceptor antibody when the cells were pulsed with the antibody before the culture, whereas continuous presence of the antibody never triggered the B cells, and resulted in the inhibition of B-cell triggering mediated by TRF (to be published elsewhere). Therefore, this discrepancy in the effect of the antiserum may be explained by the dose of antiserum present in the critical period of B-cell triggering. Figure 1 shows that, in *in vivo* conditions the B-cell responses were greater at lower concentration (1:20) than

at higher concentration (1:10), and an optimal concentration of antibody was required to trigger the B cells. This may be due to the generation of similar experimental conditions *in vivo* to those in the antibody-pulsing of B cells *in vitro*. However, this notion does not necessarily exclude other possibilities such as difference in effect of antibodies of various classes contained in the antiserum or difference in threshold of various B-cell subsets (antigen-primed B cells as opposed to non-primed B cells) to be stimulated by the antibody.

Although further investigation is required, such an allo-antiserum containing anti-TRF-acceptor antibody may provide us with the experimental strategy necessary for characterization of the TRF-acceptor site on B cells and for analysis of the fine mechanism of B-cell triggering through this site.

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Phylogenetic tracing of Ia genes

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The *I* region of the mouse *H-2* complex has been subdivided into five subregions (*A*, *B*, *J*, *E*, *C*) on the basis of serological and functional analyses of intra-MHC (major histocompatibility complex) recombinant strains¹. However, no serologically detectable specificities nor *I* genes have been localized to the *I-E* subregion of the *H-2*^b and *H-2*^s haplotypes. Attempts to identify *I-E* gene products of these haplotypes by chemical analyses have also been unsuccessful^{2–3}. Thus, the *H-2* complex of these haplotypes seems not to express *I-E* subregion genes. In this case, alloantisera raised in '*I-E*-negative' strains of mice against '*I-E*-positive' mouse cells might contain, in addition to the usual alloantibodies, antibodies reactive with conserved portions of *I-E* molecules inherited from a hypothetical primordial gene (*I-E*₀) which may have existed long before the speciation of contemporary animals. Such antibodies would show extensive cross-reactions with the *I-E* homologues of various species of animals. Interspecies cross-reactions of anti-Ia mouse alloantibodies have indeed been observed. Although such a cross-reaction was first found between mouse I-A and rat Ia-homologues, subsequent studies indicated much broader and more frequent interspecies cross-reactions of anti-I-E antibodies^{4–8}. We have now expanded the demonstration of such interspecies cross-reactions to submammalian vertebrates. The possible basis of these unusual cross-reactions and its phylogenetic implication will be discussed.

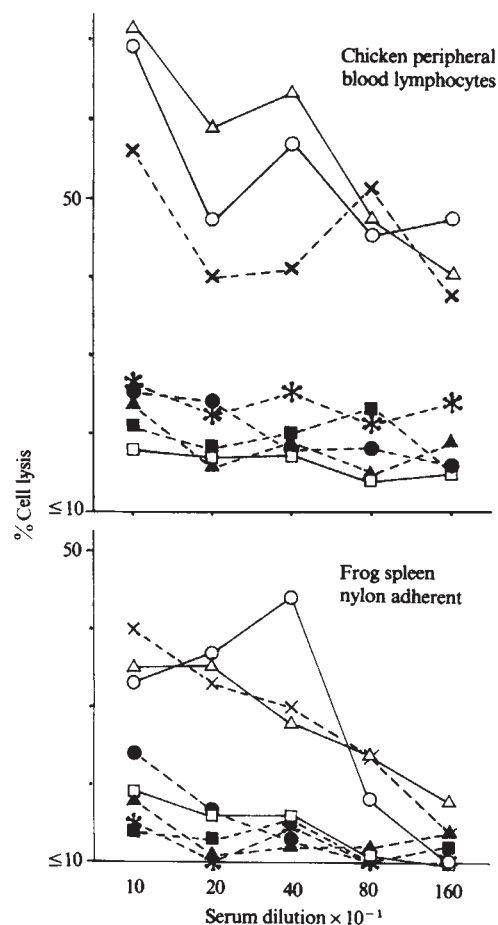


Fig. 1 Cross-reaction of anti- I^k mouse alloantibody with lymphocytes of submammalian species. Reactivity of the antiserum was assessed by two-stage Trypan blue cytotoxicity assay using rabbit complement. When necessary, rabbit complement was pre-absorbed with erythrocytes of the testing animal. Absorption of the antiserum with mouse spleen cells was performed by incubating the 1:10 dilution of the serum with 1×10^6 cells per ml for 1 h on ice. The completion of the absorption was confirmed by cytotoxicity assay on cells of the absorbing strain. Frog spleen cells were selected for adherence to nylon fibre in an attempt to enrich Ia-positive cells. Cytotoxicity curves of an A.TH anti-A.TL antiserum absorbed with: no cells (○), B10.BR (□), B10.D2 (●), B10 (△), B10.A (▲), B10.A(2R) (■), B10.A(3R) (*) and B10.A(4R) (×).

The present study used an A.TH anti-A.TL antiserum (serum 201) which is specific for the entire *I* region of the *k* haplotype. This antiserum showed cross-reactivity on lymphocytes of various mammalian species (data not shown). Further examination of the cross-reactivity of serum 201 on chicken and frog (*Rana catesbeiana*) lymphocytes revealed that the cross-reac-

tion extended even to submammalian species (Fig. 1). The cytotoxicity of the antiserum on these cells was not due to contaminating natural antibodies, as B10.BR ($H-2^k$) cells completely absorbed the cytotoxic antibodies in both cases (Fig. 1). Likewise, B10.D2 and B10.A cells absorbed the antibodies whereas B10 cells did not. Absorption of serum 201 with B10.A(2R) and with B10.A(3R) cells eliminated the cross-reactive antibodies, placing the gene encoding the cross-reactive antigen between the *I-J* and *D* regions (Fig. 1, Table 1). This result was corroborated by the failure of B10.A(4R) cells to absorb the cross-reactive antibodies. These results were identical to those observed for a variety of mammalian lymphocytes.

To confirm these findings, mouse antisera raised against various non-mammalian vertebrates such as chicken, frog and fish (*Carassius carassius*) were tested for reactivity on mouse lymphocytes. As shown in Table 2, all three antisera raised in I^s mice against non-mammalian lymphocytes showed cross-reactions on B10.BR cells. Reactivity of these antisera on A.TL and B10.S(9R) cells located the gene encoding the cross-reactive antigen between the *I-B* and *D* regions. These results not only confirmed the specificity of the cross-reactions detected by serum 201 but also indicated that even fish lymphocytes express molecules cross-reactive with *I-E/C^k* gene products.

It has already been shown in several mammalian species that the cross-reactive antigens are homologues of murine Ia antigens—glycoproteins consisting of two polypeptide chains (molecular weights 35,000 and 28,000) which are encoded by MHC-linked genes⁶⁻¹⁰. Thus, it seems quite likely that the

Table 2 Cross-reactions of mouse xenoantisera raised against submammalian lymphocytes on allogeneic mouse spleen cells

Target cells	H-2 complex								Killing by		
	K	A	B	J	E	C	S	D	A.TH α-chicken	A.TH α-frog	A.TH α-fish
A.TH	s	s	s	s	s	s	s	d	—*	—	—
A.TL	s	↗k	k	k	k	k	k	d	+	+	+
B10.S	s	s	s	s	s	s	s	s	—	—	—
B10.BR	k	k	k	k	k	k	k	k	+	+	+
B10.A	k	k	k	k	k	d	d	d	+	+	+
B10.S(9R)	s	s	?	↗k	k	d	d	d	+	+	+

* <10% above complement background.

† >25% above complement background.

cross-reactive antigens of other species are also homologues of the murine Ia molecules. The *I-A*- and the *I-E*-subregion genes in the mouse seem to have essentially overlapping functions. Although we do not know the exact nature of the functions of these genes, the *I-E* gene seems to be functionally dominant in the mouse, as judged from the extent of the alloantigenic polymorphism and the number of *Ir* genes mapped to these subregions. Such functional dominance of the *I-A* subregion in this species may account for the apparent non-catastrophic loss of *I-E* antigens in some mouse strains. From these studies, the origin of the *I-E* gene has been traced back at least to fish. This cross-reactive anti-Ia antibody may thus prove to be a powerful probe with which to study the evolutionary significance of Ia molecules and Ia-bearing cells in the immune system.

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Table 1 Mapping of the cross-reactive antigen in the mouse by absorption study

Mouse strain of absorbing cells	H-2 complex								Absorption of the cross-reactive antibody
	K	A	B	J	E	C	S	D	
B10.BR	k	k	k	k	k	k	k	k	+
B10.D2	d	d	d	d	d	d	d	d	+
B10	b	b	b	b	b	b	b	b	—
B10.A	k	k	k	k	k	d	d	d	+
B10.A(2R)	k	k	k	k	k	d	d	d	+
B10.A(3R)	b	b	b	b	↗k	d	d	d	+
B10.A(4R)	k	k	↗b	b	b	b	b	b	—

See Fig. 1 legend for details.

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Measurement of circulating prostacyclin

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Prostacyclin (PGI_2) is a potent inhibitor of platelet aggregation and a vasodilator that is synthesized from prostaglandin endoperoxides by vascular endothelial and smooth muscle cells^{1,2}. Its action on platelets is mediated by cyclic AMP³⁻⁵. Circulating PGI_2 has been detected through its inhibitory effect on the accumulation of platelets on collagen strips superfused with blood from anaesthetized and heparinized cats⁶ and rabbits⁷ and this led to the suggestion that PGI_2 is released continuously into blood passing through the lungs and functions as a circulating hormone that regulates platelet aggregability⁶⁻⁹. Because of its chemical instability, no more sensitive method of detecting circulating PGI_2 has been developed and there is doubt over whether PGI_2 is present in more physiological conditions^{10,11}. Although 6-keto-PGF_{1 α} , the stable non-enzymatic breakdown product of PGI_2 , has been assayed in human^{12,13} and rabbit^{14,15} plasma, uncertainty over the fraction present as PGI_2 in the circulation has limited the value of the results obtained. Finally, it has been suggested that 6-keto-PGE₁, a metabolic product of PGI_2 or 6-keto-PGF_{1 α} that can inhibit platelet aggregation^{16,17}, may also function as a circulating hormone¹⁸. To resolve these uncertainties, we have now developed a sensitive bioassay for PGI_2 -like compounds in blood. The results indicate that fresh arterial blood from physiologically normal rabbits contains insufficient PGI_2 or 6-keto-PGE₁ to affect platelet function.

Our assay is based on measurement of the increases in platelet ^3H -labelled cyclic AMP that occur on addition of platelets containing ^3H -labelled adenine nucleotides to blood. The ^3H -labelled cyclic AMP formation attributable to PGI_2 -like compounds in the blood was identified using an antibody that bound them. Inclusion of 1 mM 3-isobutyl-1-methylxanthine (IBMX) in the reaction mixture to inhibit platelet cyclic AMP phosphodiesterase enhanced the sensitivity of the assay about 10-fold. After addition of labelled platelets to citrated rabbit blood containing 1 mM IBMX, the increase in platelet ^3H -labelled cyclic AMP was linear with respect to time for 0.5–1.0 min, whether PGI_2 was present or not, thus providing a measure of platelet adenylate cyclase activity. When blood was preincubated at 37 °C for >1 h to remove any endogenous PGI_2 , IBMX increased the platelet ^3H -labelled cyclic AMP content from a basal value of $0.024 \pm 0.002\%$ of platelet ^3H to $0.070 \pm 0.004\%$ in 0.5 min (means $\pm$ s.e.m., $n = 30$). Double reciprocal plots of the additional increases caused by exogenous PGI_2 against the PGI_2 concentration ($10\text{--}1,000 \text{ pmol ml}^{-1}$) were linear, suggesting a single population of functional PGI_2 receptors. Computer analysis using a weighted least squares method¹⁹ gave a K_m value for PGI_2 of $29.9 \pm 2.6 \text{ pmol ml}^{-1}$ and a $V_{\max}$ for the PGI_2 -stimulated conversion of platelet ^3H -labelled adenine nucleotides to cyclic AMP of $3.7 \pm 0.1\%$ in 0.5 min (means $\pm$ s.e.m. from five experiments). For 6-keto-PGE₁, a K_m of $101 \pm 25 \text{ pmol ml}^{-1}$ and a $V_{\max}$ of $3.2 \pm 0.6\%$ were obtained (means $\pm$ s.e.m. from four experiments). However, when using the formation of ^3H -labelled cyclic AMP to assay amounts of PGI_2 or 6-keto-PGE₁ less than 5 pmol, we have found it most convenient to calculate experimental values from linear regressions of $\log [\Delta^3\text{H}\text{-labelled cyclic AMP}]$ on $\log [\text{PGI}_2]$ or $\log$

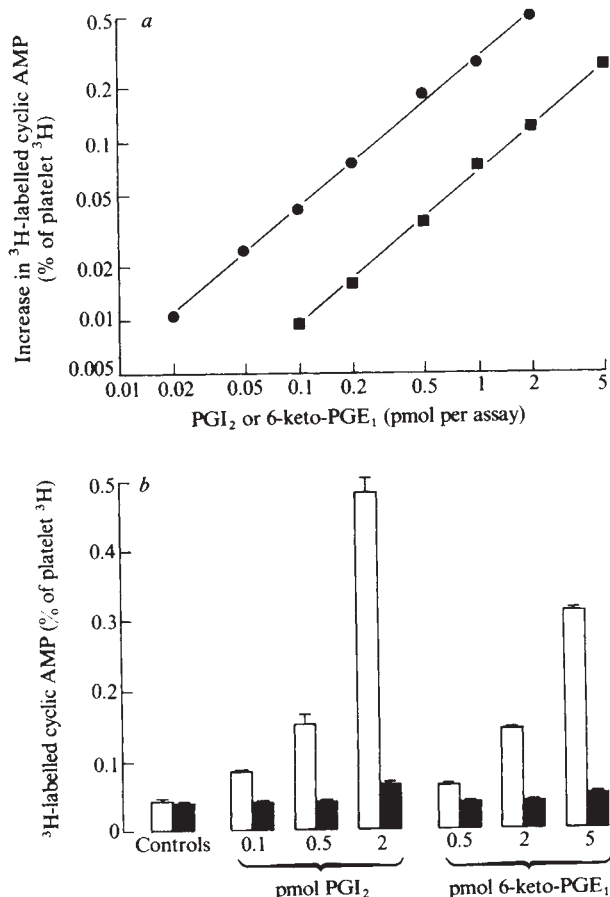


Fig. 1 Increases in ^3H -labelled cyclic AMP in labelled platelets added to blood containing IBMX and either PGI_2 or 6-keto-PGE₁; antagonistic effects of antibody prepared against 6-keto-PGF_{1 α} . Washed rabbit platelets were prepared using blood from pentobarbital-anaesthetized animals by a modification of the method of Ardlie *et al.*³¹. After the first wash the platelets were resuspended at $2.5 \times 10^9 \text{ ml}^{-1}$ in Ca^{2+} -free Tyrode's solution containing 5 mM PIPES buffer, pH 6.5, and were incubated with $2 \mu\text{M}$ [2,8- ^3H]adenine (34 Ci mmol^{-1} , ICN) for 90 min at 20–22 °C. Almost complete uptake of the ^3H -adenine occurred. The labelled platelets were then isolated by centrifugation and resuspended at $2.5 \times 10^9 \text{ ml}^{-1}$ in Tyrode's solution containing 0.35% (w/v) bovine serum albumin, 5 mM HEPES buffer, pH 7.4, and an apyrase concentration ($\sim 30 \mu\text{g ml}^{-1}$) capable of degrading 100 μM ADP at $0.1 \mu\text{mol min}^{-1}$ at 37 °C. This suspension was stored at 37 °C in a sealed tube and was usable for up to 3 h. Blood (13.5 ml) for assay of added prostaglandin standards was withdrawn from the central artery of the ear of an anaesthetized rabbit into a syringe containing 1.5 ml of 3.8% (w/v) trisodium citrate and was incubated in a sealed tube at 37 °C for 1 h to allow time for complete breakdown of any endogenous PGI_2 . Incubation mixtures (0.5 ml) were then constituted as follows: 0.4-ml samples of preincubated citrated blood were mixed with 0.5 μmol of IBMX in 0.04 ml of 0.154 M NaCl and with 0.02 ml of 0.154 M NaCl with or without γ -globulin (60 μg) from rabbits immunized against 6-keto-PGF_{1 α} (ref. 10). Prostaglandin standards (0–5 pmol) in 0.02 ml of 0.139 M NaCl containing 9.4 mM Na_2CO_3 (PGI_2) or of 0.154 M NaCl (6-keto-PGE₁) were then added and exactly 0.1 min later 0.04 ml of the suspension of labelled platelets ($1\text{--}1.5 \mu\text{Ci } ^3\text{H}$) was introduced. Incubations were continued for 0.5 min at 37 °C, at which time the reactions were stopped by addition of 1.0 ml of ice-cold 15% (w/v) trichloroacetic acid and 1,000 d.p.m. of ^{14}C -labelled cyclic AMP (28 mCi mmol^{-1} , Amersham). Each incubation was performed in triplicate. The acidified samples were centrifuged and the acid extracts applied to columns (15 $\times$ 0.7 cm) containing 1.5 g alumina (WN-3, Sigma) that had been washed with 10% (w/v) trichloroacetic acid. The columns were then washed with 9 ml of 10% (w/v) trichloroacetic acid, 9 ml of water and 2 ml of 0.2 M ammonium formate (pH 6.0) and cyclic AMP was eluted with a further 3 ml of 0.2 M ammonium formate³². The eluates were applied to columns containing 1.5 ml (packed volume) of Dowex 50 resin (Bio-Rad AG 50W-X8, 100–200 mesh, H^+ form). These columns were then washed with 6 ml of 1 mM potassium phosphate buffer, pH 7.3, and cyclic AMP was finally eluted in a further 9 ml of the same buffer which was lyophilized and counted for ^3H and ^{14}C by liquid scintillation. After correction for the recovery of ^{14}C -labelled cyclic AMP (50–70%), platelet ^3H -labelled cyclic AMP was expressed as a percentage of the total platelet ^3H . **a**, log/log plots of the increases in platelet ^3H -labelled cyclic AMP caused by different amounts of PGI_2 (●) or 6-keto-PGE₁ (■) present in blood samples also containing IBMX. **b**, ^3H -labelled cyclic AMP (mean values $\pm$ s.e.m., $n = 3$) found in labelled platelets incubated with rabbit blood containing IBMX and the indicated amounts of PGI_2 or 6-keto-PGE₁, either without (unshaded) or with (shaded) antibody. The results shown in **a** and **b** are from different experiments.

Table 1 Arterial concentrations of PGI₂ after intravenous injection of PGI₂ or angiotensin II

Compound injected	Time of PGI ₂ measurement (min)	Arterial PGI ₂ (pmol ml ⁻¹)	PGI ₂ remaining in blood (% of that injected)
PGI ₂ (1 nmol per kg)	-5	0.06 ± 0.01(4)	—
	+2	1.55 ± 0.18(4)	9.7 ± 1.1
	+5	0.19 ± 0.02(4)	0.9 ± 0.1
	+10	0.09 ± 0.01(4)	0.2 ± 0.1
Angiotensin II (5 nmol per kg)	+2	7.55 ± 0.37(4)	—
	+5	1.53 ± 0.37(3)	—
	+10	0.26 ± 0.03(3)	—
	+30	0.03 ± 0.01(3)	—

Values for PGI₂ are means ± s.e.m. of the number of unanaesthetized male rabbits indicated in parentheses. The rabbits received single intravenous injections of 3–5 µM PGI₂ in a solution containing 0.139 M NaCl and 9.4 mM Na₂CO₃ or of 20 µM angiotensin II in 0.154 M NaCl. Arterial blood (13.5 ml) for measurement of the effects of standard amounts of PGI₂ on labelled platelets (as in Fig. 1a) was taken from each rabbit 60–90 min before injection of compound and further 2.7-ml samples for immediate assay of circulating PGI₂ were taken at the times indicated with respect to injection of compound (from progressively more proximal segments of the central arteries of the ears). The per cent of injected PGI₂ remaining in the blood (means ± s.e.m.) was calculated on the approximate assumption that this compound was homogeneously distributed throughout a blood volume of 65 ml per kg. In control experiments, injections of vehicle did not affect arterial PGI₂ levels. Addition of angiotensin II (5–50 pmol) to assays had no effect on the increase in platelet ³H-labelled cyclic AMP caused by PGI₂ (2 pmol).

[6-keto-PGE₁] (Fig. 1a); for these regressions r^2 usually exceeded 0.99. The smallest amount of PGI₂ added to 0.5-ml assay mixtures (see Fig. 1 legend) that significantly increased platelet ³H-labelled cyclic AMP above the values obtained with IBMX alone was 0.02 pmol (average increase 0.009% of the platelet ³H). With <5 pmol per assay, amounts of 6-keto-PGE₁ four- to fivefold larger than PGI₂ were required to induce equivalent increases in platelet ³H-labelled cyclic AMP (Fig. 1a).

The γ-globulin fraction from rabbits immunized with 6-keto-PGF_{1α} conjugated to bovine serum albumin has been shown to block the inhibition of platelet aggregation by PGI₂ (ref. 10). We found that inclusion of 60 µg of a comparable antibody preparation in assay mixtures containing rabbit citrated blood prevented the increases in platelet ³H-labelled cyclic AMP caused by ≤0.5 pmol of PGI₂ and inhibited the effects of 2 pmol of PGI₂ by >90% (Fig. 1b). Antibody to 6-keto-PGF_{1α} also suppressed the increases in platelet cyclic AMP caused by ≤5 pmol of 6-keto-PGE₁. That these actions of the antibody preparation were due to its ability to bind PGI₂-like compounds rather than to a nonspecific effect on platelet adenylate cyclase activity was established by the observation that its effects were blocked by addition of >100 pmol of 6-keto-PGF_{1α} to the assay. To distinguish between PGI₂ and 6-keto-PGE₁, use was made of a marked difference in their stability in citrated blood. Assays (for example, Fig. 2) showed that the activity of PGI₂ added to citrated rabbit blood (pH 7.6–7.8) decreased exponentially with a half life of 9.3 ± 0.6 min (mean ± s.e.m., $n = 6$), whereas that of 6-keto-PGE₁ declined with a half life of >40 min.

To measure PGI₂-like compounds in circulating rabbit blood, 2.7 ml was rapidly withdrawn from the central artery of the ear into a syringe containing 0.3 ml of 3.8% (w/v) trisodium citrate. Citrated blood (0.4 ml) was then pipetted into tubes holding 0.5 µmol of IBMX with antibody (three tubes) and without antibody (three tubes). Labelled platelet suspension was added immediately and the mixtures (0.5 ml) were incubated for 0.5 min at 37 °C before extraction of ³H-labelled cyclic AMP (see Fig. 1 legend). All six incubations were completed within 2.5 min of arterial puncture, so that breakdown of PGI₂ had little effect on the results. The presumptive PGI₂ present in freshly taken blood was calculated from the difference between the mean values for the platelet ³H-labelled cyclic AMP formed in the presence and absence of antibody and that formed in preincubated blood from the same animal on addition of standard amounts of PGI₂ and the same preparation of labelled

platelets. Assays were performed on fresh arterial blood from 27 conscious rabbits (New Zealand White, 2.5–3.5 kg). The observed mean value for the formation of ³H-labelled cyclic AMP in labelled platelets added to the fresh blood was in each case diminished by antibody to 6-keto-PGF_{1α}, but this decrease was only statistically significant in 11 individual animals ($P < 0.05$, one-sided t -test), indicating that blood concentrations of PGI₂-like compounds were close to the limit of sensitivity of the method. Estimates of the mean arterial presumptive PGI₂ were made using groups of animals and the values obtained after correction for dilution by anticoagulant were 0.050 ± 0.007 pmol ml⁻¹ in 17 male rabbits and 0.066 ± 0.017 pmol ml⁻¹ in 10 female rabbits (means ± s.e.m.). Both mean values are significantly different from zero ($P < 0.001$ and <0.0025, respectively, in one-sided t -tests), but no significant difference between the sexes was detected. As addition of antibody did not decrease the cyclic AMP formed when labelled platelets were added to blood samples that had been preincubated at 37 °C for 30 min ($P > 0.1$; t -test, $n = 6$), the small but significant amount of PGI₂-like material present before preincubation of these samples was probably PGI₂.

We have also assayed the PGI₂-like compounds in rabbit arterial blood at various times after rapid intravenous injection of PGI₂ at 1 nmol per kg (Table 1). Approximately 90% of the injected PGI₂ had disappeared from the circulation after 2 min and 99% after 5 min. When arterial blood taken 2 min after injection of PGI₂ was incubated at 37 °C (see Fig. 2), the assayable activity disappeared with a half life of 8.3 ± 0.3 min (mean ± s.e.m., $n = 3$), indicating the presence of PGI₂ rather than 6-keto-PGE₁ in the circulation. Intravenous injection of angiotensin II has been shown to lead to the appearance of a circulating PGI₂-like substance in both cats²⁰ and dogs^{21,22}. We have observed the same phenomenon in rabbits; 2 min after injection of angiotensin II at 5 nmol per kg, the arterial concentration of presumptive PGI₂ reached 7.55 ± 0.37 pmol ml⁻¹ and then declined rapidly (Table 1). The *in vitro* half life of the material present in the circulation after 2 min was 7.4 ± 0.5 min (mean ± s.e.m., $n = 3$), indicating that it was probably PGI₂ and certainly not 6-keto-PGE₁.

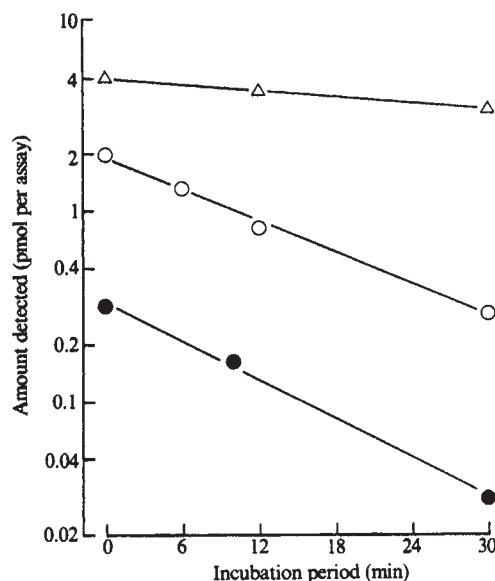


Fig. 2 Stability of PGI₂ and of 6-keto-PGE₁ in rabbit blood. Samples (0.4 ml) of citrated blood (pH 7.7) that had been preincubated for 60 min at 37 °C were mixed with 2 pmol of PGI₂ or 5 pmol of 6-keto-PGE₁. After further incubation at 37 °C for the indicated periods, IBMX and labelled platelets were added and the increase in platelet ³H-labelled cyclic AMP measured over 0.5 min. The PGI₂ or 6-keto-PGE₁ remaining was calculated from the effects of standard amounts of these prostaglandins added to samples of the same blood 0.1 min before the labelled platelets (see Fig. 1a). Samples of freshly drawn arterial blood from a rabbit injected 2 min previously with PGI₂ at 1 nmol per kg were assayed for PGI₂-like activity immediately and after incubation at 37 °C for 10 and 30 min. The decay of PGI₂ (●), 6-keto-PGE₁ (△) and of the PGI₂-like activity present in blood after injection of PGI₂ (○) are plotted on a semi-logarithmic scale.

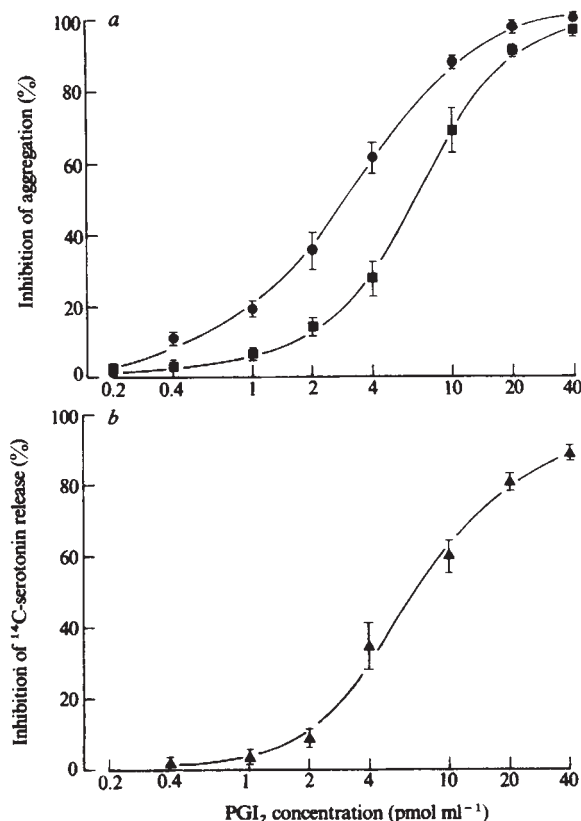


Fig. 3 Inhibition of rabbit platelet function by PGI₂. *a*, ADP-induced platelet aggregation. Incubation mixtures (1 ml) comprising 0.85 ml of citrated plasma containing 4.25×10^8 platelets and PGI₂ at the concentrations indicated were stirred for 0.5 min at 37 °C in an aggregometer (Payton Associates) before addition of ADP. The per cent inhibitions by PGI₂ of the decreases in absorbance caused by 0.75 μM ADP (●) and 2 μM ADP (■) after stirring for a further 0.5 min are shown (means $\pm$ s.e.m., $n = 5$ and 6, respectively). Control decreases in absorbance (means $\pm$ s.e.m.) were 0.134 ± 0.015 with 0.75 μM ADP and 0.208 ± 0.019 with 2 μM ADP. *b*, Collagen-induced release of serotonin. Incubation mixtures (1 ml) comprising 0.8 ml of citrated plasma containing 4×10^8 platelets (labelled by preincubation with 0.5 μM ¹⁴C-serotonin and the indicated concentrations of PGI₂) were stirred for 0.5 min before addition of collagen (50 μg ml⁻¹). After stirring for a further 2 min, 0.2 ml of 9% (w/v) paraformaldehyde was added, the samples were centrifuged and the release of platelet ¹⁴C-serotonin into the supernatant was measured; this amounted to $57 \pm 5\%$ in controls without PGI₂ ($n = 6$). The per cent inhibitions of release (means $\pm$ s.e.m.) are shown (▲).

To determine whether the small amounts of PGI₂ normally found in rabbit arterial blood could affect platelet function, we studied the sensitivity of the aggregation of platelets by ADP and of the release of platelet serotonin by collagen to inhibition by PGI₂ in rabbit citrated platelet-rich plasma (Fig. 3). As preliminary experiments showed that preincubation with PGI₂ for 0.5 min gave an optimal inhibition, this period was used subsequently. With a concentration of ADP that caused maximum aggregation (2 μM), an IC₅₀ for PGI₂ of 7 pmol ml⁻¹ was obtained (Fig. 3a), similar to the value reported by Whittle *et al.*²³. Almost the same concentrations of PGI₂ were required to inhibit the release of serotonin by collagen at 50 μg ml⁻¹ (Fig. 3b). With sub-optimal doses of ADP, PGI₂ was more effective (Fig. 3a), as expected in view of the ability of ADP to inhibit platelet adenylate cyclase⁵. However, no inhibition was observed with PGI₂ concentrations < 0.4 pmol ml⁻¹. 6-Keto-PGE₁ was approximately one-fifth as effective as PGI₂ in inhibiting ADP-induced platelet aggregation.

Our experiments show that rabbit arterial blood contains trace amounts of a compound that shares three properties with PGI₂, namely the capacity to activate platelet adenylate cyclase, an affinity for an antibody to 6-keto-PGF_{1α} and lability in whole blood. However, the values we have obtained for this presumptive PGI₂ are one to two orders of magnitude lower than those implied by the collagen strip superfusion technique⁷ or by two

studies in which 6-keto-PGF_{1α} levels were measured in rabbit serum¹² and plasma¹⁴. Although the collagen strip method did not allow accurate quantitation of circulating PGI₂, antibody to PGI₂ enhanced the deposition of platelets from rabbit arterial blood to about the same extent as infusion of PGI₂ at 1 ng ml⁻¹ (2.8 pmol ml⁻¹) into venous blood depressed platelet deposition⁷. This suggests that arterial PGI₂ concentrations of this order were present. A possible explanation of the discrepancy between these results and ours is that various features of the superfusion technique may stimulate PGI₂ synthesis, for example, the embolization of platelet aggregates from the collagen strip into the lungs. Intravenous injection of an air embolus has been shown to stimulate the release of PGI₂ in the cat⁸. The high 6-keto-PGF_{1α} content of rabbit serum¹² may reflect synthesis of PGI₂ by white cells from PGH₂ released by stimulated platelets²⁴. Although one group¹⁴ has reported a mean 6-keto-PGF_{1α} concentration of 0.75 ng ml⁻¹ (2 pmol ml⁻¹) in rabbit plasma, another group¹⁵ obtained values for plasma 6-keto-PGF_{1α} of less than 0.1 ng ml⁻¹ (0.3 pmol ml⁻¹).

We believe our technique provides the most sensitive and accurate method yet available for measuring blood PGI₂. The results obtained show that in physiologically normal rabbits, the arterial concentration of PGI₂ is an order of magnitude too low to affect platelet function. Thus, our findings do not support the concept, based partly on experiments with rabbits⁷, that PGI₂ is a circulating hormone that regulates platelet aggregability⁶⁻⁸. Of course, the absence of significant PGI₂ from the normal circulation does not preclude its release in large amounts and distant action in conditions that provoke its synthesis by a substantial fraction of the pulmonary or peripheral vascular bed, as is apparently observed after injection of angiotensin II. However, in view of the fairly rapid clearance of PGI₂ from the circulation that we and others²⁵ have observed, we suggest that the synthesis and action of PGI₂ are most often likely to be part of a local response to a local stimulus. Such PGI₂ synthesis may have important functions in limiting the extension of haemostatic plugs and thrombi²⁶⁻²⁹ and in the dispersal of platelet aggregates trapped by the lungs and other organs³⁰.

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The phosphatidylinositol cycle and the regulation of arachidonic acid production

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An increase in the metabolism of phosphatidylinositol occurs in a wide variety of tissues by the action of specific ligands¹⁻³. In platelets, the interaction of thrombin with its receptor initiates the degradation of phosphatidylinositol by the action of a specific phospholipase C (refs 4-8). In normal conditions of stimulation, the resultant 1,2-diacylglycerol is rapidly and completely phosphorylated to phosphatidic acid⁴⁻¹¹. The formation of phosphatidic acid precedes the release of arachidonic acid from the phospholipids of stimulated platelets⁵. This early appearance of phosphatidate might result in the initial production of arachidonic acid and lysophosphatidic acid by the action of a phospholipase A₂ specific for phosphatidate¹². Phosphatidate/lysophosphatidate could induce calcium-gating¹³⁻¹⁵ and subsequently stimulate phospholipases of the A₂-type⁸, that degrade phosphatidylcholine, phosphatidylethanolamine and a further fraction of phosphatidylinositol⁶. Alternatively, the lysophosphatidate produced may serve as a substrate for the transfer of arachidonate directly from other phospholipids^{16,17} to form new phosphatidate which in turn can release more arachidonate. Overall, such a sequence would be equivalent to phospholipase A₂ activation of other phospholipids. Our present data indicate that when the release of arachidonic acid is completely inhibited by cyclic AMP or quinacrine, phosphatidic acid is redirected entirely to phosphatidylinositol and there is no production of arachidonate. In these conditions, the availability of calcium might be profoundly restricted. The correlation in platelets of a phosphatidylinositol by a specific phospholipase A₂ might suggest that these phenomena are applicable to activations in other cell systems.

Phosphatidylinositol is not the only phospholipid that contributes to the production of arachidonic acid in stimulated platelets^{4-10,18}. Phosphatidylcholine and phosphatidylethanolamine also release arachidonic acid by the action of phospholipase A₂ activities⁸. Both lysophosphatidylcholine and lysophosphatidylethanolamine have recently been found in stimulated platelets^{10,18}. In a similar way, lysophosphatidic acid is also produced in intact platelets that have been prelabelled with ³²P and stimulated with thrombin (Fig. 1). Thrombin is very effective in producing phosphatidic acid^{4-6,19} and lysophosphatidic acid¹⁹, whereas ionophore A23187 forms virtually no phosphatidic acid^{4,5} or lysophosphatidic acid (Fig. 1). Calcium ions enhance the thrombin-induced formation of phosphatidic acid as well as of lysophosphatidic acid (Fig. 1). We have described elsewhere the existence of a specific phospholipase A₂ which is present in platelet membranes and which specifically degrades phosphatidate¹². This enzyme activity (K_m 20 μ M) is most active at pH 7.0, requires Ca²⁺ (10 μ M) for maximal activity and is inhibited by quinacrine¹². The existence and specific properties of this enzyme suggest a possible important role in the production of arachidonic acid in stimulated platelets. This phosphatidate-specific phospholipase A₂ has distinctly different properties from those of the phospholipases A₂ that degrade phosphatidylethanolamine and phosphatidylcholine⁸, as its activity does not depend on the presence of detergents, alkaline pH or high concentration of Ca²⁺.

Phosphatidate is a key intermediate in the phosphatidylinositol cycle¹⁻³. In this cycle, four consecutive enzyme activities are involved in the degradation and resynthesis of phosphatidylinositol (phosphatidylinositol-specific phospholipase C; 1,2-diacylglycerol kinase; CTP-phosphatidate: cytidyl

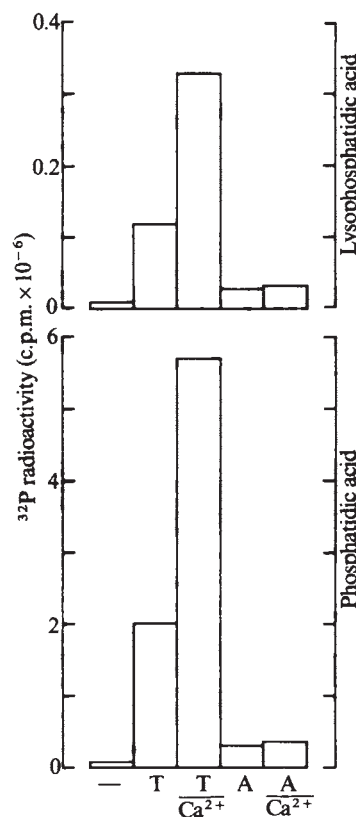


Fig. 1 Effect of thrombin and ionophore A23187 on the formation of phosphatidic acid and lysophosphatidic acid in platelets. Horse platelets were labelled with ³²P-orthophosphate after separation from one (550 ml) unit of blood as described previously^{5,6}. Platelets were then resuspended in 10 ml of buffer (134 mM NaCl, 15 mM Tris-HCl pH 7.4, 1 mM EGTA, 5 mM glucose), 5 mCi of ³²P-orthophosphate were added and the platelets incubated at 37 °C for 2 h. After centrifugation and resuspension, the final concentration of platelets was 1 × 10⁹ per 0.5 ml, which was the volume used for the assays. Samples (0.5 ml) were incubated with thrombin (T) (1 unit ml⁻¹) or ionophore A23187 (A) (1 μ M) ± calcium chloride (3 mM) in both cases, for 10 min at 37 °C. Lipid extraction and chromatographic separation of lipids have been detailed elsewhere^{5,6}. Phosphatidic acid and lysophosphatidic acid were separated by a TLC method which uses oxalate-impregnated plates as described before¹².

transferase; CDP-1,2-diacylglycerol-inositol phosphatidyl transferase). To study the effects of calcium on the phosphatidic acid and phosphatidylinositol of stimulated platelets, platelets were labelled with ³²P-orthophosphate and resuspended in an EGTA-containing buffer. If those platelets are then incubated with quinacrine and stimulated with thrombin, the release of arachidonic acid is completely blocked but phosphatidic acid is formed as a consequence of the degradation of phosphatidylinositol⁶. After an initial period, the label in phosphatidate decreases while the labelling of phosphatidylinositol increases (Fig. 2). This increased conversion of phosphatidate to phosphatidylinositol is blocked by the addition of ionophore A23187 plus calcium ions (Fig. 2). In this case, there is a further accumulation of labelled phosphatidic acid while the increased labelling of phosphatidylinositol is completely blocked (Fig. 2). These results indicate that calcium inhibits the resynthesis of phosphatidylinositol from phosphatidic acid after thrombin stimulation (Fig. 2)¹⁰. In fact, Ca²⁺ has a direct inhibitory action on the enzymes involved in the resynthesis process (CTP-phosphatidate: cytidyl transferase and CDP-1,2-diacylglycerol-inositol phosphatidyl transferase)^{20,21}. These data indicate that the phosphatidylinositol cycle can actively function in the presence of quinacrine, which completely blocks the production of arachidonic acid from all platelet phospholipids⁶. Calcium, on the other hand, interrupts the phosphatidylinositol cycle and phosphatidate accumulates (Fig. 2).

Cyclic AMP inhibits the 'release reaction' of platelets as well as aggregation⁴. The action of cyclic AMP on platelet enzymes has been variously ascribed to the inhibition of the conversion of arachidonic acid to cyclooxygenase metabolites²², the production of arachidonic acid from phospholipids^{4,23-27} and the formation of phosphatidic acid^{4,5}. All these actions ultimately reduce the production of arachidonate or its conversion to active cyclooxygenase products. We previously described the action of cyclic AMP in reducing phosphatidic acid to an inhibition of phospholipase C⁷. Further studies now reveal that the phosphatidylinositol cycle is not inhibited by cyclic AMP despite the profound reduction in the quantity of phosphatidate produced. Figure 3 describes the action of cyclic AMP on the reactions related to the increased turnover of phosphatidylinositol in platelets prelabelled with ³²P-orthophosphate. Cyclic AMP seems substantially to increase the rate of conversion of phosphatidate to phosphatidylinositol, thereby decreasing the steady state concentration of phosphatidate. As we are proposing that the production of arachidonic acid might be related to the formation of phosphatidate, this could serve as the basis for the cyclic AMP-induced inhibition of arachidonate production. In the presence of quinacrine, which completely blocks the formation of arachidonic acid from various phospholipids^{6,12}, thrombin greatly increases the breakdown and resynthesis (turnover) of phosphatidylinositol as shown by increased labelling of ³²P-phosphatidylinositol (Fig. 3). These data indicate that the integrity of the phosphatidylinositol cycle is maintained in

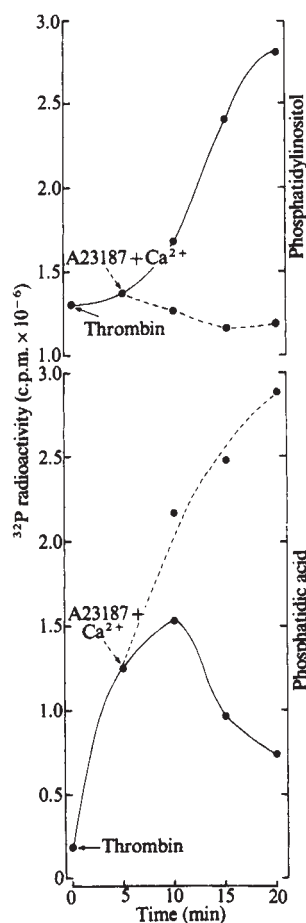


Fig. 2 Effect of ionophore A23187 plus calcium ions on phosphatidylinositol and phosphatidic acid of platelets pretreated with quinacrine and thrombin. Platelets prelabelled with ³²P-orthophosphate were obtained as for Fig. 1. Samples (0.5 ml) were incubated with quinacrine for 5 min at 37°C and then thrombin (1 unit ml⁻¹) was added for different periods of time as indicated. After 5 min treatment with thrombin, in some assays (----) ionophore A23187 (1 μM) plus calcium chloride (3 mM) were added. Other details as for Fig. 1.

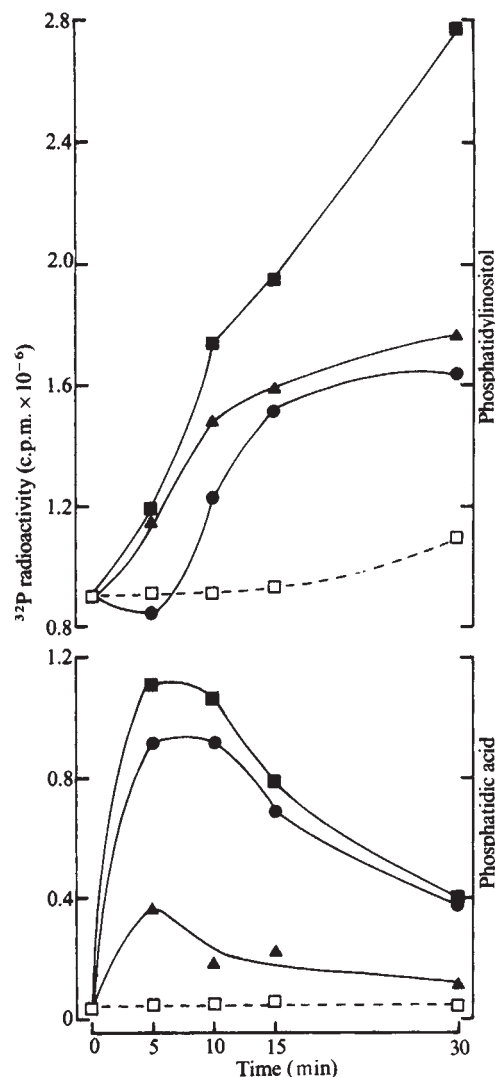


Fig. 3 Effect of thrombin on phosphatidylinositol and phosphatidic acid of platelets pretreated with cyclic AMP or quinacrine. Preincubations with quinacrine (0.75 mM) (■) or dibutyl cyclic AMP (1 mM) (▲) were for 5 min and thrombin (1 unit ml⁻¹) was then added for different periods of time as indicated. ●, Assays containing only thrombin; □, control assays. Phosphatidic acid was separated on TLC⁵ and phosphatidylinositol was separated on formaldehyde-impregnated paper^{5,6}. Other details as for Fig. 1.

conditions (cyclic AMP or quinacrine) in which the specific release of arachidonic acid induced by thrombin is completely blocked.

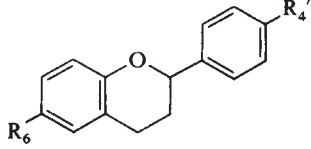
The information presented here indicates that calcium interrupts the phosphatidylinositol cycle¹⁻³ and leads to accumulation of the intermediate product, phosphatidic acid. In stimulated platelets a specific phospholipase A₂ released arachidonic acid from the phosphatidate produced¹², with the consequent appearance of lysophosphatidic acid. This phosphatidate-lysophosphatidate interconversion might be important in the subsequent and specific mobilization of arachidonic acid from phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol⁶. In thrombin-stimulated platelets cyclic AMP enhances the overall turnover of the phosphatidylinositol cycle by increasing the rate of conversion of phosphatidic acid to phosphatidylinositol, and thus inhibits the release of arachidonic acid from various phospholipids.

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Table 1 Structure–activity relationship of a selection of halogen-substituted flavans against rhinovirus type 1B

		
R ₆	R ₄ '	IC ₅₀ (μM)
H	H	0.046
F	H	0.020
Cl	H	0.050
Br	H	0.019
H	F	0.018
H	Cl	0.039
H	Br	0.036
F	F	0.068
Cl	Cl	0.007
Br	Br	0.010
I	I	0.043

4',6-Dichloroflavan (BW683C), a new anti-rhinovirus compound

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Derivatives of flavan have been synthesized as chemical intermediates, but the only reported biological action is the ability of certain alkyl and alkoxy derivatives to lower blood cholesterol concentrations¹. It was therefore surprising to discover that flavan itself (Table 1) is a highly effective inhibitor of the replication of certain serotypes of rhinovirus, and that a simple derivative, BW683C (4',6-dichloroflavan), is the most potent antiviral compound yet reported. The present work examines the antiviral activity of flavan derivatives with a view to selecting the compound most suitable for trial in volunteers infected with a common cold virus.

4',6-Dichloroflavan, which is new to the chemical literature, has been prepared by methods used for the synthesis of substituted flavans^{2–5}. It is a colourless crystalline solid, m.p. 101 °C, soluble in water only to the extent of 1 mg l⁻¹ at room temperature.

Antiviral activity was detected *in vitro* by means of plaque inhibition tests^{6,7} with monolayers of M-HeLa cells^{8,9} infected with rhinovirus 1B. Activity was measured by plaque reduction assays in which doubling concentrations of compound were incorporated into the overlay medium. Plaque counts, expressed as a percentage of the control value, were plotted against the logarithm of the compound concentration, to yield a dose-response line from which the IC₅₀ value could be determined. The IC₅₀ values for BW683C and several analogues are shown in Table 1. Flavan (R₆ = R₄' = H), with an IC₅₀ of 0.046 μM, is one of the least active of the compounds tested. The activity is generally increased by the presence of a single halogen substituent, and more so by the presence of two chlorine atoms, with the most active compound tested being BW683C which, with an IC₅₀ of 0.007 μM, is some six times more potent than the parent compound. The IC₉₀ of BW683C was 0.02 μM.

For any agent to be effective in the prophylaxis or treatment of the common cold, it must be active against a high proportion of rhinovirus serotypes. There are at least 89 serotypes, the most prevalent being 1A, 1B, 2, 4, 15, 29, 30 and 31 (ref. 10). IC₅₀ values were obtained for BW683C against 19 serotypes (Table 2). Seven of the eight most prevalent serotypes were inhibited, although they varied considerably in sensitivity. The sensitivity of the other 11 serotypes was also variable, but was sufficient to suggest that the compound may be clinically useful.

In tissue culture tests 4',6-dichloroflavan did not inhibit the replication of other RNA viruses, including bunyavirus, coronavirus, equine rhinovirus, influenza virus (NWS strain), measles virus, poliovirus (Sabin 1), Semliki Forest virus, Sindbis virus and respiratory syncytial virus. It also failed to inhibit the DNA viruses adenovirus type 5 and herpesvirus type 1.

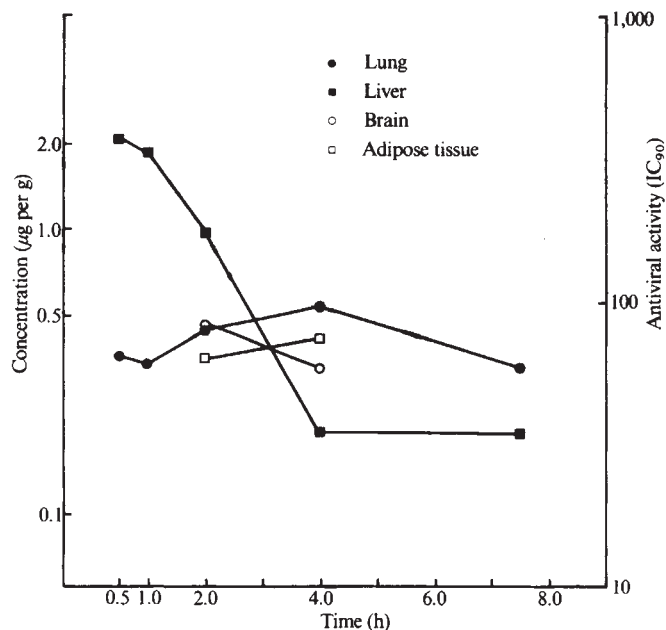


Fig. 1 Tissue concentrations of BW683C determined by gas-liquid chromatography. Tissue homogenate (1 ml) was mixed with 0.5 ml ethylene glycol/water/2 M citric acid (2:2:1) and 5 ml hexane. The mixture was shaken for 30 min and centrifuged. The hexane layer was collected and mixed with 1 ml of a mixture of ethylene glycol/1 M Na₂CO₃ (1:9), shaken again and centrifuged. The upper layer was collected, dried in a stream of N₂ and the residue was dissolved in a small volume of toluene.

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Table 2 Sensitivity of rhinoviruses to BW683C

Serotype	IC ₅₀ (μM)	Serotype	IC ₅₀ (μM)
1A*	0.013	13	0.66
1B*	0.007	14	Inactive
2*	0.04	15*	0.17
3	10.10	16	0.02
4*	Inactive	18	0.29
5	Inactive	19	0.81
8	8.00	21	1.70
9	0.011	29*	0.008
12	0.15	30*	52.00
		31*	0.013

* Serotypes described by Roebuck¹⁰ as common.

Preliminary studies were carried out on the absorption and tissue distribution of 4',6-dichloroflavan in rats after its administration at 5 mg per kg by gavage as a suspension of finely ground particles in aqueous methylcellulose. Samples of plasma and selected tissues were collected at intervals up to 7.5 h after dosing, and unchanged compound was determined by analysis of tissue extracts by gas-liquid chromatography with electron capture detection or by specific ion monitoring using a mass spectrometer. Highest concentrations were found in liver (Fig. 1); peak concentrations of ~7.3 μM, some 350 times the IC₉₀, were observed at 0.5 h, and these declined rapidly. Concentrations of compound well in excess of the IC₉₀ value were also detected in plasma, lung, brain and adipose tissue, and the time of peak compound concentrations in lung samples seemed to occur between 2 and 4 h after dosing; compound concentrations in excess of the IC₉₀ level could still be found in lung 7.5 h after dosing. These results indicate that 4',6-dichloroflavan is adequately absorbed from the gastrointestinal tract, and that compound concentrations well above those required for maximum antiviral activity *in vitro* can be attained at doses as low as 5 mg per kg. The apparent persistence of this compound implies that infrequent administration may suffice to maintain effective antiviral concentrations *in vivo*. The above findings were supported by autoradiography of rats, which received ³H-BW683C as an aqueous suspension by gavage, and were killed 2 or 4 h later.

When BW683C was added to HeLa cells in culture no cytotoxic effects were observed at drug concentrations up to 3.6 μM, the limit of aqueous solubility and many times the IC₉₀ and IC₅₀ values determined with rhinovirus 1B. In the rat, the LD₅₀ after subcutaneous administration was ~300 mg per kg; animals showed no ill effects after oral administration at doses up to 1 g per kg or intraperitoneal administration at doses up to 700 mg per kg. BW683C showed no evidence of mutagenicity when tested in the presence or absence of a liver microsome preparation by the method of Ames¹¹.

Preliminary studies showed that BW683C interacted directly with virus, reducing infectivity by about 0.5 log unit, but it was not a typical contact inactivator because inactivation did not increase with time. The possible binding of compound to virus particles was investigated by mixing tritiated compound with virus and centrifuging the mixture through a 15–45% sucrose gradient; radioactively labelled compound was found in the virus peak.

In a time-of-addition study, the compound was added at hourly intervals throughout a single growth cycle of rhinovirus 1B in M-HeLa cells. Maximum antiviral activity was obtained when the compound was added to the culture at the same time as the virus. Although some antiviral activity was observed when addition was delayed for 1 h or longer, it was no greater than that obtained by adding compound at the end of the cycle.

Further experiments with ³H-uridine-labelled virus indicated that the compound does not interfere with adsorption of the virus to the cell, nor with the uncoating or entry of the viral RNA into the cell. However, viral RNA synthesis was inhibited by the presence of the compound. BW683C therefore seems to bind to

the virus and to inhibit some stage of viral replication immediately following entry of the viral RNA into the cell.

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Mutation of gene encoding regulatory polypeptide of aspartate carbamoyltransferase

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In our studies on the regulation of the expression of the genes for the biosynthesis of arginine and pyrimidines in *Escherichia coli*, we discovered that an aspartate carbamoyltransferase (ATCase) synthesized *in vitro* from our λ transducing phage λOTC3 lacked substrate cooperative interactions and was insensitive to feedback inhibition by CTP. We show here that these abnormal properties result from a mutation in the gene for the regulatory polypeptide chain of ATCase. We believe this to be the first report of a mutation in the gene for this regulatory chain.

ATCase catalyses the first reaction specific to pyrimidine biosynthesis, the formation of *N*-carbamoyl-L-aspartate from L-aspartate and carbamoylphosphate. In *E. coli*, ATCase activity is modulated by feedback inhibition by CTP, the end product of the pyrimidine pathway, and activation by ATP, product of the parallel purine pathway¹. Because *E. coli* ATCase exhibits the various types of regulatory interactions characteristic of allosteric proteins, it has been considered a model system and has become the most extensively investigated regulatory enzyme^{2,3}. It differs, however, from most other allosteric proteins in that its catalytic and regulatory functions can be physically separated. Native ATCase, with a molecular weight (MW) of 300,000, consists of two trimeric subunits which possess catalytic activity but are insensitive to the allosteric effectors and three dimeric subunits which are catalytically inactive but bear the effector binding sites^{4–6}. Thus, the completely different catalytic (C) and regulatory (R) chains (respective MWs 34,000 and 17,000) form a 2C₃:3R₂ structure. Zn²⁺ ions, one per regulatory chain, are required to stabilize this quaternary structure^{7–9}.

The zinc-binding domains on the regulatory subunits participate in the R:C bonding domains essential for the allosteric interactions¹⁰. The gene *pyrB* coding for the catalytic chain of ATCase lies at 96 min on the *E. coli* chromosome^{11,12}, but the localization of the gene for the regulatory chain has been hampered by the failure to obtain mutations affecting this gene. However, a thorough investigation of their biosynthesis^{13,14}

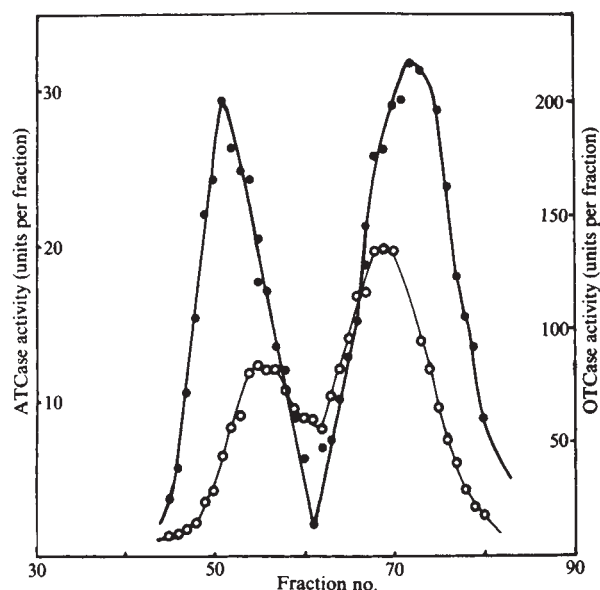


Fig. 1 Analysis of aspartate carbamoyltransferase (ATCase) from strain PR7 by gel filtration on a column of Sephadex G-200. A 1-ml sample (containing 1,200 units and 10 mg protein) of crude extract of PR7 cells grown on minimal medium and extracted as described previously^{21,22} was filtered on a 2.5 × 40-cm column of Sephadex G-200 equilibrated with 50 mM potassium phosphate buffer (pH 7.5). Elution was performed using the same buffer and 1.5-ml fractions were collected. *E. coli* and *Streptococcus faecalis* ornithine carbamoyltransferase (OTCase), exhibiting respective apparent MWs of 140,000 and 250,000 (ref. 23), were used as protein markers for calculating ATCase molecular weight. ATCase activity was determined by measuring the formation of ¹⁴C-carbamoylaspartate from ¹⁴C-carbamoylphosphate. The complete (600 μl) assay mixture containing 50 mM Tris-acetate (pH 8.0), 20 mM L-aspartate, 5 mM ¹⁴C-carbamoylphosphate (0.03 mCi mmol⁻¹) and enzyme sample was incubated at 37 °C for 15 min. The reaction was stopped by the addition of 400 μl 0.25 M trichloroacetic acid. After 5 min boiling to destroy the non-reacted carbamoylphosphate, ¹⁴CO₂ formed was eliminated by bubbling air for 5 min. A 0.5-ml sample was counted in a model LS-100 Beckman liquid scintillation spectrometer in the presence of a scintillation cocktail containing 4 parts of Triton X-100 to 6 parts of a solution containing 0.5% 2,5-diphenyloxazole and 10% naphthalene in dioxane. A blank obtained by omitting the enzyme sample was subtracted from the results. OTCase was assayed as described previously²⁴. Units of enzyme activity are μmol of product formed per h. ●, ATCase activity; ○, OTCase activity.

suggested the existence of an operon encoding both types of ATCase chains in the order catalytic first and regulatory next. Moreover, in *Salmonella typhimurium*, which has a very similar ATCase¹⁵, *pyrB* deletions have been found to produce no detectable regulatory subunit¹⁶. The present study characterizes a λ transducing phage carrying the genetic determinants of an *E. coli* ATCase with modified regulatory subunits. This establishes the proximity of the genes encoding both types of chain. In agreement with this conclusion, the *pyrB* region of *E. coli* was

cloned recently as a 2,800-base pair DNA fragment encoding both subunits of ATCase (J. R. Wild & G. A. O'Donovan, personal communication). In agreement with Wild *et al.* (see accompanying report)¹⁷ in the present study we reserve the designation *pyrB* for the gene encoding the C chain of ATCase and use the symbol *pyrI* for the R chain of this enzyme.

λ Bacteriophages transducing the contiguous *E. coli* genes *argI* (encoding ornithine carbamoyltransferase) and *pyrB*¹⁸ have been used as a source of template DNA for *in vitro* synthesis of ATCase¹⁹. However, the enzyme synthesized using one of these DNA sources, λ0TC3, was insensitive to CTP inhibition and exhibited hyperbolic aspartate saturation curves. Accordingly, bacteriophage λ0TC3 was introduced into strain KMBL1510 G4, a thermoresistant derivative of strain KMBL1510 (F⁻ *pyrB* :: Mu-1 cts62) that produces no detectable amount of either ATCase subunit (G. Hervé and B. Perbal, personal communication).

ATCase activity in the resulting strain PR7, was only slightly sensitive to CTP and lacked cooperative interactions with aspartate. When cell extracts of strain PR7 were submitted to gel filtration on a column of Sephadex G-200 (Fig. 1), two ATCases were observed with respective MWs of 300,000 and 100,000. Using the same experimental conditions, practically all the ATCase activity of a wild-type strain for this enzyme was present as a single activity peak with a MW of 300,000, and negligible amounts as the 100,000 MW species. Both ATCase forms of strain PR7 exhibited hyperbolic aspartate saturation curves (Fig. 2b, c), the half-saturation concentration of the larger form ($[S]_{0.5} = 6.5$ mM) being significantly lower than that of the smaller form ($[S]_{0.5} = 23$ mM). Whereas the larger ATCase of strain PR7 exhibited a reduced response to CTP and ATP, the smaller ATCase of this strain was totally insensitive to these effectors.

Comparison of the two ATCases of PR7 with the wild-type holoenzyme and its catalytic subunit (Table 1) suggested that the smaller ATCase (trimeric C₃) was identical with the wild-type catalytic subunit. The larger species corresponded to a 300,000-MW holoenzyme formed from this same catalytic subunit associated with a modified regulatory subunit. This interpretation is supported by the following experiments. (1) Treatment of the larger ATCase from PR7 with *p*-hydroxymercuribenzoate²⁰ yielded a smaller ATCase (MW 100,000) having all the properties of the wild-type catalytic subunit. (2) A single ATCase species (MW 300,000) displaying sigmoidal aspartate saturation and effector responses similar to those of wild-type ATCase (Table 1 and Fig. 2a, d) could be reconstituted by combining the catalytic trimer of strain PR7 with purified wild-type regulatory subunit. (3) A similar reconstitution could be achieved *in vivo* by introducing the phage λ0TC3 into strain 30S0U4 Hfr *thi pyrB*) harbouring a missense mutation in the *pyrB* gene but producing normal regulatory chain (Fig. 2e). These results indicate that phage λ0TC3 encodes a wild-type catalytic ATCase chain and a modified regulatory chain.

The precise nature of the modification affecting the regulatory chains of ATCase remains to be determined. Nevertheless, heteroduplex analysis (not shown) of phage λ0TC3 indicates

Table 1 Comparison of aspartate carbamoyltransferases (ATCases) used in the present study

ATCase species	Wild-type (native)	Wild-type (catalytic subunit)	Modified (larger MW)	Modified (smaller MW)	Reconstituted holoenzyme (PR7 catalytic + wild-type regulatory subunits)
Molecular weight	300,000	100,000	300,000	100,000	300,000
Subunit structure	2C ₃ :3R ₂	C ₃	2C ₃ :3R ₂	C ₃	2C ₃ :3R ₂
Aspartate kinetics	Sigmoidal	Hyperbolic	Hyperbolic	Hyperbolic	Sigmoidal
$[S]_{0.5}$ value for aspartate	18 mM	25 mM	6.5 mM†	23 mM†	14 mM
CTP inhibition*	77%	No effect	11%	No effect	38%
ATC activation*	360%	No effect	38%	No effect	256%

The $[S]_{0.5}$ value was determined at pH 8 as described in Fig. 1 legend.

* Inhibition by 2 mM CTP and activation by 5 mM ATP in the presence of 5 mM aspartate.

† $[S]_{0.5}$ values calculated from Lineweaver-Burk plots of the data from Fig. 2b, c.

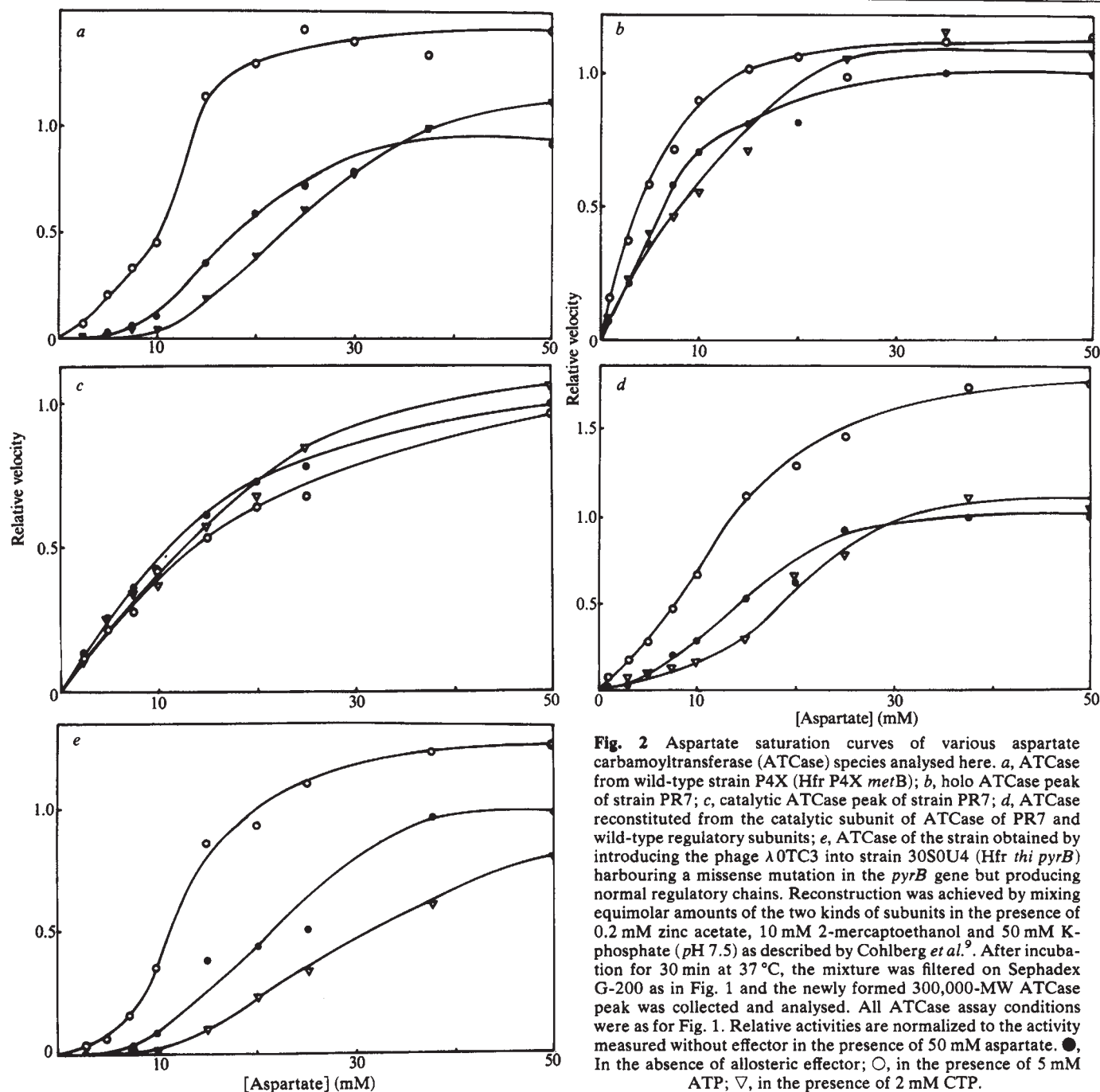


Fig. 2 Aspartate saturation curves of various aspartate carbamoyltransferase (ATCase) species analysed here. *a*, ATCase from wild-type strain P4X (Hfr P4X *metB*); *b*, holo ATCase peak of strain PR7; *c*, catalytic ATCase peak of strain PR7; *d*, ATCase reconstituted from the catalytic subunit of ATCase of PR7 and wild-type regulatory subunits; *e*, ATCase of the strain obtained by introducing the phage $\lambda 0TC3$ into strain 30S0U4 (Hfr *thi pyrB*) harbouring a missense mutation in the *pyrB* gene but producing normal regulatory chains. Reconstruction was achieved by mixing equimolar amounts of the two kinds of subunits in the presence of 0.2 mM zinc acetate, 10 mM 2-mercaptoethanol and 50 mM K-phosphate (pH 7.5) as described by Cohlberg *et al.*⁹. After incubation for 30 min at 37 °C, the mixture was filtered on Sephadex G-200 as in Fig. 1 and the newly formed 300,000-MW ATCase peak was collected and analysed. All ATCase assay conditions were as for Fig. 1. Relative activities are normalized to the activity measured without effector in the presence of 50 mM aspartate. ●, In the absence of allosteric effector; ○, in the presence of 5 mM ATP; ▽, in the presence of 2 mM CTP.

that the modified gene lies at the extremity of a 7,300-base pair chromosomal segment inserted into the phage genome. It is therefore possible that a short 3'-terminal portion of the gene has been deleted during the formation of phage $\lambda 0TC3$. This hypothesis is being evaluated by cloning and sequencing the modified regulatory gene. Interestingly, the C-terminal end of the regulatory chain with its zinc-binding sites lies at the interface between the regulatory and catalytic chains¹⁰. Even short 3'-terminal deletions affecting the regulatory chain may thus impair the R:C binding domain. This might be the case for the modified ATCase encoded by $\lambda 0TC3$ which, as suggested by the presence of two ATCase forms in extracts of strain PR7, may be impaired in holoenzyme assembly.

Further work is being done on the purification and characterization of the heavy ATCase species encoded by $\lambda 0TC3$. A detailed analysis of this unusual enzyme and its comparison with the wild-type enzyme may be vital for determining the role of the R:C domain of bonding in the allosteric interactions of ATCase. For example, it will be of interest to investigate whether the modified regulatory subunits have altered proper-

ties towards zinc. Although the ATCase distribution shown in Fig. 1 could not be modified following growth in the presence of high Zn^{2+} concentrations, preliminary experiments suggest that reaggregation of the two kinds of subunits of strain PR7 can be promoted *in vitro* provided that a high enough Zn^{2+} concentration is present.

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A mutation in the catalytic cistron of aspartate carbamoyltransferase affecting catalysis, regulatory response and holoenzyme assembly

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We describe here a mutation in the gene encoding the catalytic subunit of aspartate carbamoyltransferase (ATCase, *pyrB*) which produces an enzyme retaining catalytic activity as holoenzyme ($2C_3:3R_2$) and catalytic trimer (C_3) but which shows neither cooperative substrate kinetics nor nucleotide effector response. Furthermore, the holoenzyme assembly seems quite fragile in that the enzymatic activity is recovered only partially as holoenzyme following growth of *Escherichia coli* in the presence of zinc. In contrast, the enzyme from wild-type *E. coli* strain E63 is recovered almost entirely in its dodecameric form in identical conditions. The gene encoding the mutant *pyrB* was isolated from a λ darg1⁺*pyrB*⁺ transducing phage (obtained from N. Glansdorff). This mutation differs from other previously reported mutations affecting the catalytic subunit^{1,2} in that significant catalytic activity is retained but homotropic and heterotropic communication is lost³.

A 6.0-kilobase fragment was isolated from purified λ DNA⁴, restricted with the endonuclease *Pst*I (Bethesda Research Laboratory)⁵, and cloned on to plasmid pBR322 (ref. 6). The recombinant plasmid pPB-h204 (plasmid-pyrimidine-B cistron-holoenzyme-strain 204) was transformed into *E. coli* WR38, which contains a Mu insertion⁷ in the chromosomal *pyrB* and does not produce catalytic or regulatory polypeptides as determined by immunoassay with specific antisera (W.D.R., unpublished observations). This plasmid encodes both the catalytic (*pyrB*) and the regulatory (*pyrI*) polypeptides of ATCase. [A mutation in the cistron encoding the regulatory polypeptide is described in the accompanying report by Feller *et al.*⁸. The cistron is designated *pyrI* in agreement with Bachmann (personal communication).] The synthesis of ATCase in the transformed strain, WR38-h204, is repressed by growth in uracil, so that the cloned fragment contains the operator-promoter region as well as both ATCase cistrons. The catalytic activity recovered from this strain is distributed as ~40% holoenzyme with a molecular weight (M_r) of 300,000 and 60% as the catalytic trimer with a M_r of 100,000. Both forms of ATCase from this mutant lack cooperative homotropic kinetics for aspartate, do not respond to the allosteric effectors ATP and CTP, and seem to have altered affinities for aspartate.

ATCase is the archetype of allosteric enzymes because: (1) both its substrates, carbamoyl phosphate and aspartate, produce

Table 1 ATCase holoenzymes from wild-type *E. coli* strain E63 and mutant *E. coli* strain WR38-h204

	Wild-type	Mutant
[S] _{0.5} (mM aspartate)	5.5 mM	2.0–2.5 mM
% Activity + CTP	21%	97%
% Activity + ATP	166%	105%
ATCase activity at pH 7.0		
ATCase activity at pH 8.4	3.1	0.51
Shape of velocity-substrate curve	Sigmoidal	Hyperbolic

ATCase assay mixtures of 2.0 ml volume contained 40 mM potassium phosphate, pH 7.0, 3.6 mM dilithium carbamoyl phosphate, pH 7.0, 5 mM potassium aspartate, pH 7.0, for the wild-type and 2.5 mM for the mutant (approximate [S]_{0.5} values), and enzyme (holoenzyme fractions of G-200 eluate, Fig. 2). CTP or ATP was absent from control tubes and present at a concentration of 2 mM when inhibition (CTP) or activation (ATP) was measured. The control activity, that is, the activity in the absence of effector, is set at 100%. ATCase activity was assayed by measuring the amount of carbamoyl aspartate formed in 30 min at 30 °C as previously described²³. Carbamoyl aspartate production was determined at 466 nm. Specific activity is expressed as nmol carbamoyl aspartate formed per min per mg protein. Specific activity of ATCase was determined in conditions in which carbamoyl aspartate formation was proportional to extract concentration and time. The value obtained for the ratio of ATCase specific activity at pH 7.0/ATCase specific activity at pH 8.4 may be used as an index for the presence or absence of cooperativity between catalytic sites for aspartate binding according to the methods of Kerbiriou and Hervé¹¹. A ratio of <1.0 signifies the absence of cooperativity; one of ≥2.0 reflects cooperative homotropic interactions. Fractions from beneath the holoenzyme peak (Fig. 2) were used to estimate this ratio.

positive homotropic interactions between catalytic sites as evidenced by the sigmoidal dependence of activity on substrate concentrations^{9,10}, (2) substrate binding is subject to positive heterotropic interactions between catalytic and regulatory sites in the presence of ATP and negative heterotropic interactions with CTP^{11–14}, and (3) the binding of CTP is subject to negative homotropic interactions between the regulatory sites¹⁵. Moreover, ATCase is unusual among allosterically regulated enzymes, in that, as with yeast phosphofructokinase¹⁶, the regulatory protein is distinct and can be physically dissociated from the catalytic subunit. The assembly of the enzyme is a cytoplasmic event¹⁷ which produces a dodecamer with six regulatory and six catalytic polypeptides (r_6c_6) associated as two separable catalytic trimers and three regulatory dimers^{18,19}. The holoenzyme may be reversibly dissociated by mild treatment with mercurials such as *p*-chloromercuribenzoate²⁰ or neohydryl²¹. The catalytic subunit (c_3) is insensitive to allosteric effectors, possesses a half-saturation concentration ([S]_{0.5}) higher for aspartate than the holoenzyme (8–10 mM compared with 5 mM) and produces a V_{max} that is two- to fourfold higher⁹. The separate regulatory subunit (r_2) has no catalytic activity although ATP and CTP may still be bound²⁰. On reassociation of the holoenzyme after removal of the mercurial by zinc replacement dialysis in the presence of dithiothreitol, the original catalytic and regulatory properties of the native enzyme are re-established²².

ATCase was prepared from *E. coli* wild-type strain E63 and the transformed mutant strain WR38-h204 by the methods of Wild *et al.*²³. Several pertinent properties of the wild-type and mutant ATCases are compared in Table 1. We must emphasize three points regarding these data. (1) The mutant ATCase is not subject to allosteric regulation by either ATP or CTP at subsaturating concentrations of aspartate (1–5 mM). Minimal effector response was observed when velocity-substrate plots were examined over the range of 0.5 mM to 50 mM aspartate (<5% variation throughout the range). As noted by Gerhart²⁴, such effects are due only to the direct competition for any phosphate-containing compound. (2) The apparent [S]_{0.5} for aspartate of the mutant enzyme is significantly lower (2.0–2.5 mM) than the wild-type requirements (5.5 mM). (3) The homotropic kinetic responses of the mutant enzyme are dramatically reduced or abolished in the mutant enzyme as shown by the lack of sigmoidal dependence of activity on aspartate concentration (Fig. 1) even when plotted according to Eadie-Hofstee²⁵ (see insert Fig. 1). Thus, there is no apparent cooperativity from 0.5 to 10 mM (20% to fivefold [S]_{0.5}, respectively). In addition, the

ratio of ATCase activity at pH 7.0 and 8.4 may be used as an index of the presence or absence of homotropic interactions between catalytic sites as first described by Kerbiriou and Hervé¹¹. A ratio of <1.0 signifies the absence of cooperativity and is always found for the catalytic subunits alone, whereas a ratio of ≥ 2.0 reflects cooperative homotropic interactions¹¹. We have verified these observations in similar experiments (K.F.F. unpublished observations). By this criterion, the ATCase from strain WR38-h204 seems to lack cooperativity.

Figure 2 shows an analysis of the M_r values of the ATCases from mutant and wild-type enzymes in the presence of zinc. A single large molecular weight form ($\sim 300,000$) was observed from the wild-type extract. In contrast, more than half of the enzymatic activity from the mutant extract was observed as a smaller component ($M_r \sim 100,000$) which corresponds to the wild-type catalytic trimer. We have shown previously that all native ATCase from the enteric bacteria grown in sufficient zinc (20 μ M) studied thus far are recovered entirely as holoenzyme²³. Only when zinc becomes limiting or there is an assembly defect can the catalytic trimer be recovered from cell-free extracts.

The following evidence suggests that these differences are consequences of an altered catalytic subunit. (1) The catalytic trimer from the mutant enzyme shows increased affinity for aspartate compared with the catalytic trimer ($[S]_{0.5}$ of 5 mM aspartate compared with 8–10 mM) of the wild-type enzyme. (2) The M_r values of the catalytic trimers are indistinguishable, but they form clearly different holoenzymes when reconstituted with wild-type regulatory dimers (Table 2). The holoenzyme

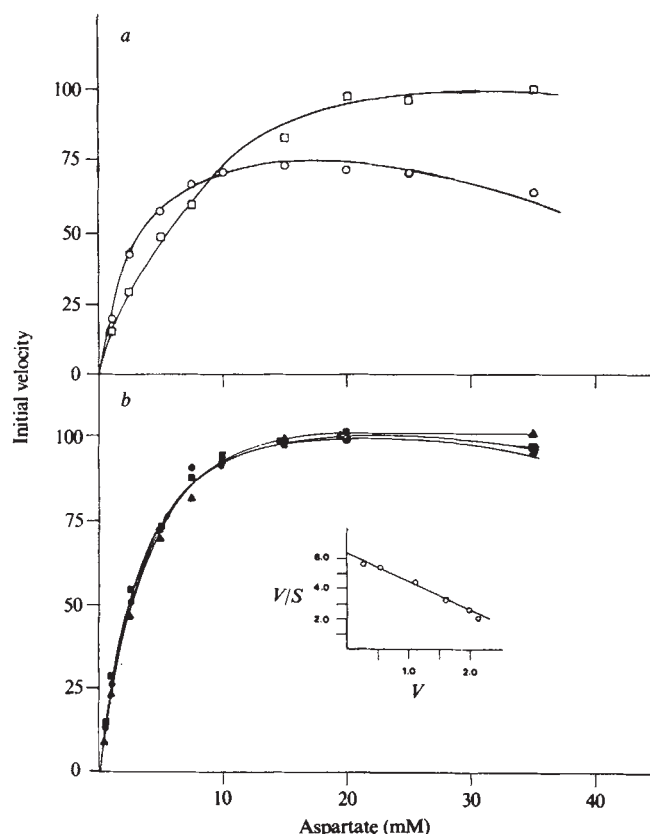


Fig. 1 Aspartate saturation curves for ATCase holoenzyme and its catalytic trimers from *E. coli* mutant strain WR38-h204. Enzyme samples were prepared as reported previously²³. Assay mixtures were as described in Table 1 legend except that the aspartate concentration was varied from 0.5 mM to 50 mM as indicated on the abscissa. CTP or ATP was absent from control tubes and present at 2 mM when inhibition (CTP) or activation (ATP) was measured. For the above comparisons initial rate values were normalized as described below. *a*, Velocity-substrate plots for aspartate of the ATCase holoenzyme (○) and its catalytic trimer (□) from the mutant. The maximum velocity for the catalytic trimers was set at 100 on the ordinate scale for convenience. *b*, Velocity-substrate plots for aspartate of the ATCase holoenzyme from the mutant in the absence of the effectors (●) and in their presence: CTP (▲) or ATP (■).

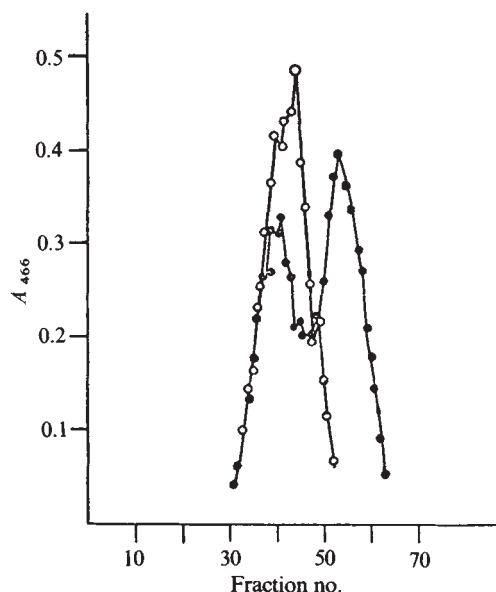


Fig. 2 Analysis of ATCase from *E. coli* wild-type E63 and mutant WR38-h204 by gel filtration on a G-200 Sephadex column; 1 ml, containing ~ 20 mg protein, of the appropriate cell-free extract was applied to the G-200 column, equilibrated previously with 40 mM potassium phosphate buffer pH 7.0, 0.1 mM dithiothreitol and 0.02 mM zinc acetate. The equilibration mixture was also used for elution. Individual fractions (abscissa) were assayed for ATCase by a spot assay. This differed from the usual assay²³ in that the final reaction volume was reduced from 2.0 ml to 0.2 ml and 30–50 mM aspartate was used. The ordinate gives absorbance at 466 nm, the wavelength for monitoring ATCase. A single wild-type peak (○) and two mutant peaks (●) are seen.

formed from wild-type regulatory dimers and mutant catalytic trimers has a M_r of $\sim 300,000$, but its regulatory and catalytic properties are those of the original mutant enzyme.

We have shown that a mutation in the *pyrB* gene, encoding the catalytic polypeptide of ATCase, can affect the homotropic and heterotropic interactions of the enzyme while maintaining catalytic activity. The reconstitution experiments with normal regulatory subunits purified from the native enzyme and catalytic subunits from both the mutant and wild-type strains prove that the catalytic polypeptide of the ATCase from strain WR38-h204 is altered. It is surprising that a mutation in the cistron encoding the catalytic polypeptide can affect such a wide range of enzymatic properties without completely disrupting the

Table 2 Catalytic trimers of ATCase from wild-type and mutant *E. coli* strains and their reconstituted holoenzyme derivatives

	Wild-type	Mutant
M_r for c_3	85,000–100,000	85,000–100,000
$[S]_{0.5}$ aspartate for c_3	8–10 mM	5 mM
M_r for holoenzyme	280,000–300,000	280,000–300,000
$[S]_{0.5}$ aspartate for holoenzyme	5.5 mM	2.0 mM
% Activity in 2 mM CTP	20	86–95
% Activity in 2 mM ATP	160	97–105

Reconstitution experiments were performed as follows. Catalytic subunits from the wild-type and mutant strains were mixed individually with excess of wild-type regulatory subunits and concentrated on an Amicon ultrafiltration system (PM-10 membrane) in the presence of 40 mM potassium phosphate buffer, pH 7.0, 0.2 mM zinc acetate, 2.0 mM dithiothreitol and 20 μ M EDTA. The reconstitution of holoenzyme with both wild-type and mutant catalytic trimers was almost 100%. Effector response was measured exactly as described for Table 1. The effector response was determined at the appropriate $[S]_{0.5}$ for each enzyme (that is 5 mM or 2.5 mM). Molecular weights were estimated using Sephadex G-200 ascending flow column chromatography. Standards used in column calibration were: RNase A, M_r 13,700; chymotrypsinogen A, 25,000; ovalbumin 45,000; aldolase 158,000; ATCase holoenzyme from *E. coli* 300,000; Blue Dextran 2000, 2×10^6 . These standard enzymes (5–20 mg of each, except ATCase) were prepared in a 2.0-ml volume of 40 mM potassium phosphate, pH 7.0, with 0.02 mM zinc acetate, 0.1 mM dithiothreitol, and applied to the column. The elution profiles of the various proteins were determined by monitoring absorbance at 280 nm. ATCase was used separately to verify the accuracy of the column at higher molecular weights. Catalytic and regulatory subunits from the wild type were prepared as described previously²¹. The catalytic subunit from the mutant was obtained from appropriate Sephadex G-200 column fractions (Fig. 2).

catalytic capabilities of the enzyme. This mutation should be compared with that affecting the regulatory polypeptide described by Feller *et al.*⁸ in the accompanying report. It is striking that the holoenzyme assembly is apparently deficient in both mutants, one affecting the catalytic subunit and the other the regulatory subunit.

One final observation regarding the catalytic mutation seems pertinent. Examination of the kinetic characteristics (Fig. 1) of the mutant (with or without ATP added) revealed that they approximate the wild-type enzyme in the presence of ATP. Indeed, the high affinity exhibited by the mutant holoenzyme for aspartate suggests that the mutant ATCase may be frozen in its activated R state²⁶. The present data suggest that strikingly similar characteristics can exist for two independent mutations in either the catalytic or regulatory cistrons of ATCase.

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Sequence dependence of the helical repeat of DNA in solution

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Considerable progress has recently been made on the fine structure of DNA. X-ray diffraction studies of crystals of oligonucleotides of defined sequences have already provided several structures at atomic resolution¹⁻⁸. Whereas all the crystals studied so far show bihelical structures with antiparallel chains and Watson-Crick-type base pairing, there is a striking range of structural variability, from the right-handed B-type to the left-handed Z-type helices. In contrast to the well developed methodology for crystal structure determination, few methods of high precision and low ambiguity are available for studies of DNA in solution. Here we report the application of the band-shift method previously developed in this laboratory⁹ to the determination of the dependence of the DNA helical repeat in solution on its nucleotide sequence.

Plasmid	Sequence	Total insert length(bp)
pBR322	:::CTGCA PstI site ACGTC:::	0
pLP119	:::CTGCATT:::AAA:::TTTCAAG:::	19
pLP140	:::CTGCATT:::AAA:::TTTCAAG:::	40
pLP144	:::CTGCATT:::AAA:::TTTCAAG:::	44
pLP219	:::CTGCACCC:::CCC:::CCCTGCAG:::	19
pLP222	:::CTGCACCC:::CCC:::CCCTGCAG:::	22
pLP225	:::CTGCAGG:::CCC:::GGGCAAG:::	25
pLP234	:::CTGCAGG:::CCC:::GGGCAAG:::	34

Fig. 1 Sequences of homopolymer tract inserts. Clones were initially screened for insert size by following the restriction endonuclease *Hae*III digest on a 6% polyacrylamide gel. For DNA sequencing, the insert containing the *Alu*I-generated restriction fragment was isolated, end-labelled, and sequenced after strand separation according to the procedures of Maxam and Gilbert²³. In most cases both strands were sequenced. No changes in plasmid sequence were observed outside the *Pst*I site.

The principle of the band-shift method has been reported elsewhere⁹. Note that the helical repeat, h^0 , obtained from the band-shift method is the number of base pairs of the sequence inserted that will increase the average linking number of the DNA in its relaxed state by one. In cases where the insert does not have an intrinsic spatial writhe, h^0 is also identical to the number of base pairs that forms a complete helical turn.

In addition to the absolute magnitudes of the helical repeats of different sequences, the band-shift method gives the relative handedness of the helices. We shall make the generally accepted assumption that a double-stranded DNA of typical sequence is a right-handed helix in solution. The band-shift method then provides directly the handedness of the inserted helical segments as well.

To extend the band-shift method to the determination of the helical repeats of DNAs of defined sequences, families of covalently closed circular DNAs containing inserts of these sequences of known lengths are needed. The method used to insert homopolymer tracts of various lengths into the *Pst*I site of pBR322, a plasmid with known nucleotide sequence¹⁰, involved tailing with terminal transferase and one of the deoxynucleoside triphosphates¹¹. On digestion with restriction endonuclease *Bam*HI, preparative gel electrophoresis allowed isolation of the desired fragments. The appropriate pairs of DNA fragments thus obtained were ligated together with T4 polynucleotide ligase and competent *Escherichia coli* cells were transformed. The nucleotide sequences in the regions containing the inserts are given in Fig. 1. In several cases, at the junctions between the plasmid DNA and the homopolymer inserts, a few unexpected base changes occurred. These changes were probably due to a low level of 3'→5' exonuclease activity in the calf thymus terminal transferase used in the construction of these plasmids.

An example of the gel electrophoretic patterns of several DNA samples relaxed in identical conditions is shown in Fig. 2a; lane 9 illustrates the band pattern observed when a single DNA, that of plasmid pLP222, is present. In lane 8, a second DNA (pLP219) shorter than pLP222 by three GC base pairs (bp) is mixed in. It can be readily seen that the band pattern of the longer plasmids shows a relative upward shift of ~0.3 times the interband spacing between topoisomers.

The set of covalently closed DNA samples prepared are all positively supercoiled in electrophoresis conditions¹², thus the faster migrating topoisomers have higher linking numbers than the slower migrating ones. Therefore an upward shift (that is, a reduction in the distance migrated) by 0.3 times the interband

Table 1 Helical repeats of $(dA)_n \cdot (dT)_n$ and $(dG)_n \cdot (dC)_n$

DNA pair		Length difference (bp)	Shift observed	Corrected shift	Linking difference deduced	Calculated h^0 (bp per turn)
$(dA)_n \cdot (dT)_n$ inserts	pLP140/pLP144	4	0.44	0.42	0.42	10
	pBR322/pLP119	19	0.00	0.91	1.91	9.9
	pLP119/pLP140	21	0.15	0.05	2.05	10.2
	pLP119/pLP144	25	0.59	0.47	2.47	10.1
	pBR322/pLP140	40	0.15	0.95	3.95	10.1
	pBR322/pLP144	44	0.59	0.37	4.37	10.1
$(dG)_n \cdot (dC)_n$ inserts	pLP219/pLP222	3	0.28	0.27	0.27	11
	pLP219/pLP225	6	0.59	0.56	0.56	11
	pLP225/pLP234	9	0.88	0.84	0.84	11
	pLP222/pLP234	12	0.18	0.12	1.12	10.7
	pLP219/pLP234	15	0.47	0.40	1.40	10.7
	pBR322/pLP219	19	0.88	0.79	1.79	10.6
	pBR322/pLP222	22	0.17	0.06	2.06	10.7
	pBR322/pLP225	25	0.47	0.35	2.35	10.6
	pBR322/pLP234	34	0.35	0.18	3.18	10.7

Because of the particular construction of the homopolymer inserts and the base changes at some of the junctions, the homopolymer tracts do not make up the total length differences (second column) except for the pairs pLP119/pLP144, pLP219/pLP222 and pLP225/pLP234. For most of the other pairs sequences other than the homopolymers make up 5–10% of the total differences. Values of h^0 calculated are those of the total inserts. When the shift (third column) was <0.2 or >0.8 of the interband spacing, accurate measurements were obtained by the use of a common internal standard. For the pair pBR322/pLP119, for example, each member of the pair was measured against pLP144, and the same upward shift of 0.59 was observed. This shows that there is no shift between pBR322 and pLP119. For corrected shift an alternative way of making the length correction is to assume that the intrinsic length dependence of the electrophoretic mobility of a covalently closed topoisomer is the same as that for a nicked circular DNA. For DNAs of sizes similar to pBR322, the reduction in the distance migrated by a nicked circular DNA per bp increment in length was measured as 3.7×10^{-3} times the average spacing between two covalently closed topoisomers that differ by 1 in their linking numbers; this value was used to make the corrections in previous experiments⁹. However, this procedure underestimated the correction, mainly because the topoisomers have migrated further than the nicked DNA, by a factor of ~ 1.2 on average. This results in a 20% underestimation. When this factor is taken into consideration, the two correction procedures give data which agree well. Note that although the absolute magnitudes of the helical repeats measured by the band-shift method are a function of the length correction factor, any differences between the helical repeats of particular sequences are not affected.

spacing agrees with a structure in which the 3-GC pair insert is in a right-handed helical form with a helical repeat of 3/0.3 or 10 base pairs per turn. The band-shift method cannot distinguish an upward fractional shift of 0.3 from a downward fractional shift of 0.7. Thus this single result by itself is equally consistent with the three GC pairs being in a left-handed helical form with a helical repeat of 3/0.7 or 4.3 base pairs per turn. Examination of several pairs of DNAs shows that consistent h^0 values are obtained only if the sense of the helical inserts is taken to be right handed. The same conclusion is arrived at with all other sequences examined.

Quantification of measurements for all pairs of DNAs were made on microdensitometer tracings of the negatives of gel photographs. A typical trace is shown in Fig. 2b to illustrate the resolution of the method. The observed shifts are given in column 3 of Table 1.

As described previously⁹, the band-shift method measures the displacement of one family of topoisomers of a DNA with an x -bp insert relative to another family of topoisomers of the same DNA without the insert. Although for small inserts the electrophoretic mobilities of the topoisomers are affected by the inserts mainly by their effects on the deviations of the linking numbers of the topoisomers from the average linking number of the DNA in its relaxed state, the lengthening of a DNA *per se* does have a small but significant effect on its mobility. Correction for this intrinsic length effect is necessary to obtain data of high accuracy. Methods for making the length corrections have been discussed previously⁹. In one, the shift for each pair of DNAs is calculated from the average of the upward shift of the longer plasmid seen with both DNAs in the positively supercoiled form and the downward shift seen with both DNAs in the negatively supercoiled form. The intrinsic length effect enhances the upward shift but reduces the downward shift, thus the averaging cancels out this effect. An example is shown in Fig. 3.

Using this method we have examined five pairs of DNAs with inserts 34–55 bp long and with sequences varying from

$(dA)_n \cdot (dT)_n$ to $(dG)_n \cdot (dC)_n$. The average length correction factor calculated for these five pairs was 4.9×10^{-3} turns per bp with a s.d. of the mean of $\pm 0.2 \times 10^{-3}$. Deviation of the correction factor calculated for any one of the five pairs from that of the mean was found to be statistically insignificant, and the average was used for all the corrections.

The values for the calculated helical repeats after corrections for the length effect are given in the last column of Table 1. Clearly, when the lengths of the inserts are progressively increased the values for the helical repeat of GC inserts rapidly approach 10.7 ± 0.1 bp per turn. This is not significantly different from the helical repeat of 10.6 ± 0.1 bp per turn found for a 58-bp segment in the *cro* gene region of phage λ when the refined length correction reported here is applied to the published results⁹. In contrast, the homopolymer $(dA)_n \cdot (dT)_n$ shows a helical repeat that is significantly different from those of the other sequences examined. The value 10.1 ± 0.1 bp per turn obtained for this sequence is the only one measured so far that agrees, within experimental error, with the classical 10-fold B-type helix of DNA.

The values measured are for the DNAs in electrophoresis buffer at room temperature. Effects of counterions and temperature on h^0 are well known but are $<1\%$ within the ranges generally considered to be physiological^{13,14}. The handedness of the helices is identical for all the sequences examined, and is presumably dextrorotatory.

For sequences that are more or less random, the average helical repeat measured by the band-shift method for DNA in solution agrees remarkably well with that measured from the nuclease digestion pattern of DNA adsorbed on solid surfaces¹⁵. The two very different methods give the same value of 10.6 ± 0.1 bp per turn. The theoretical calculation of Levitt¹⁶ also gives a similar value for the helical repeat of DNA in solution.

There is a significant discrepancy between the solution measurements and X-ray diffraction studies of DNA fibres. Although DNA fibres show polymorphism when the counter-

ions and humidities are varied, the high-humidity form for DNAs of typical sequences is the B-type structure with 10 bp per turn. Recent measurements with highly solvated fibres gave the same results¹⁷.

There are various possible reasons why the fibre results do not agree with solution measurements. One potential complication brought into focus by a crystal structure of a dodecamer is the spatial writhe of DNA^{7,8}, and the plausible effect of lateral association of DNA molecules in a fibre on this writhe. Another explanation (suggested by D. M. Crothers) is that electric dichroism measurements of DNA in solution indicate that the structure is sensitive to aggregation induced by heavy metal ions. The magnitude of the dichroism in the absence of heavy metal ions can be interpreted by the structure calculated by Levitt¹⁶ with the base pairs assuming propeller twists and a 10.6-bp periodicity. In the aggregated state, the magnitude of the transient dichroism changes to that of a B-type structure with the base pairs almost perpendicular to the helical axis. A third possibility is that packing forces in fibres or crystals tend to favour structures with integral helical repeats¹⁸. Even in highly wetted fibres, the packing forces might be significant relative to the free energy differences among different polymorphic forms.

In the case of the homopolymer sequence $(dA)_n \cdot (dT)_n$, the solution measurements agree with fibre diffraction studies. The agreement between the helical repeat of poly(dA) · poly(dT) from fibre diffraction measurements and our solution measurements provides a clue to the reason for the disagreements between other sequences. The sequence $(dA)_n \cdot (dT)_n$ seems to be unique in its inertness to environmental effects^{19–22}. Both IR and fibre diffraction studies indicate that the B-type to A-type transition which can be induced for the

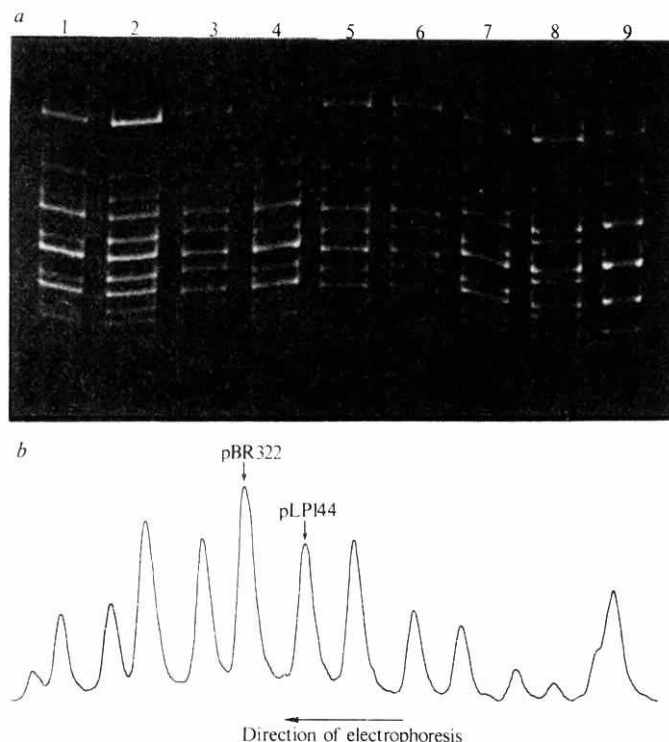


Fig. 2 *a*, Electrophoretic patterns of DNAs. DNA samples were relaxed overnight at 0°C with calf thymus topoisomerase I in 10 mM Tris-HCl pH 8, 0.2M NaCl, 0.1 mM EDTA. The reaction was stopped and the enzyme removed by extraction with neutralized phenol. Electrophoresis in 0.7% agarose was carried out at room temperature after dialysis of the DNA samples against the electrophoresis buffer (90 mM Tris-borate pH 8.3, 2.5 mM EDTA). Plasmids present in the gel lanes are: 1, pLP225/pLP140; 2, pLP140/pLP144; 3, pBR322/pLP144; 4, pBR322/pLP225; 5, pLP219/pLP234; 6, pLP219/pLP225; 7, pLP222/pLP225; 8, pLP219/pLP222; 9, pLP222. *b*, Microdensitometer tracing of the negative for the electrophoretic pattern of the pair of DNAs pBR322/pLP144 (lane 3 of *a*).

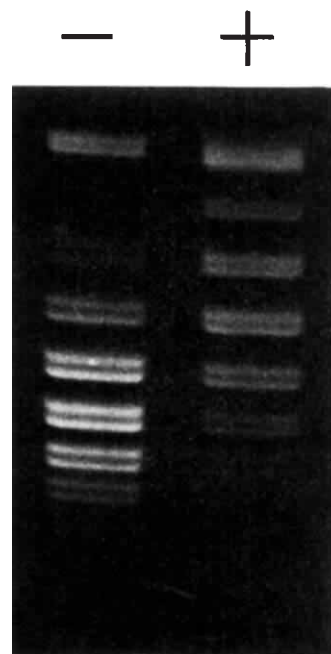


Fig. 3 Electrophoretic gel pattern of a mixture of pTR161 and pTR182, two DNAs that differ by an insert of 53 base pairs of a sequence in the *cro* gene region of phage λ ⁹. Relaxation of the DNAs was the same as described in Fig. 2 legend except that during the topoisomerase I reaction the sample on the left contained ethidium. The covalently closed DNAs in the left lane are negatively supercoiled whereas those in the right lane are positively supercoiled. The DNAs were identified by comparing the gel patterns with those of positively and negatively supercoiled marker DNAs.

other sequences does not occur for poly(dA) · poly(dT). The sensitivity of other sequences to environmental effects and packing forces may underlie their polymorphism in fibres and crystals, and cause structural differences between the condensed and soluble states.

The possible biological significance of different helical geometries associated with DNAs of different sequences has been widely discussed. The solution measurements have the advantage that the DNA is in a more physiological state and is free from packing forces. Our results clearly demonstrate that the sequence $(dA)_n \cdot (dT)_n$ has a distinct helical geometry in solution. We hope that the method described here will find increasing application in cases where the molecular geometries of particular sequences are thought to have significant biological roles.

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Sequence-dependent helical periodicity of DNA

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We have recently described a new approach to measuring the helical periodicity of random sequence DNA in solution¹. It consists of binding short, stiff pieces of DNA to various flat surfaces and using DNase I to probe the most accessible phosphodiester bonds. The differences in length of the fragments produced are measured with high precision to give the helical repeat directly. This approach has now been extended to study the periodicity of defined-sequence DNAs using another enzyme, micrococcal nuclease, as well as DNase I. We find the helical repeat of poly(dA-dT) to be 10.5 ± 0.1 base pairs (bp), a value very close to that found earlier by us for random-sequence DNA, 10.6 ± 0.1 bp, and confirmed here. Poly(dA).poly(dT) exhibits a distinctly different helical repeat, 10.0 ± 0.1 bp.

In our earlier work we used two different experimental approaches. The first used short DNA fragments of defined length, nominally 140 bp extracted from nucleosome cores, and the ³²P label incorporated beforehand at the 5' termini. This method, besides giving accurate distances between DNase I cutting sites, also enables the probability of cutting at each site along the length of the DNA fragment to be determined. The second used DNA fragments with a wide distribution of lengths, an average of ~200 bp, and the ³²P label was introduced after DNase I digestion. As a less laborious alternative, the ³²P label has now been incorporated by nick translating each DNA sample. As fragments of poly(dA-dT) and poly(dA).poly(dT) of defined length are not readily available, double-stranded fragments a few hundred base pairs long were produced by shearing samples of calf thymus DNA (Sigma, 'highly polymerized'), long poly(dA-dT) and poly(dA).poly(dT) (both BCL Boehringer) in a sonicator², which gives a broad fragment-length distribution of 200–600 bp.

Figure 1a, b and c show the results from experiments where DNA, poly(dA-dT) and poly(dA).poly(dT) were immobilized on calcium phosphate microcrystals and mica powder, then lightly digested with micrococcal nuclease or DNase I and the products fractionated by polyacrylamide gel electrophoresis^{3,4}. The autoradiographs show patterns of fine bands, each band representing single-stranded fragments differing in size by one nucleotide. The intensities of the fine bands vary, showing band maxima at regular intervals ~10 bases apart. A set of intense fine bands represents a set of fragments produced by cutting with the nuclease at two different groups of accessible phosphodiester bonds ('sites') in the same DNA molecule. The 'ladder' formed by successive groups of fine intense bands is a direct visualization by the nuclease of the helical repeat.

The average distance between nuclease cutting sites can be obtained by counting the number of fine bands between maxima and then dividing by the number of maxima traversed. The calculation can easily be carried out from the densitometer tracings of the autoradiographs shown in Figs 2 and 3. For example in Fig. 3, in the densitometer tracing of poly(dA).poly(dT) digested with DNase I, the number of fine peaks between F3 and F10 is 70, giving a value of $70/7 = 10.0$ bases.

For random-sequence DNA (Fig. 1a) the distance between DNase I cutting sites is 10.5–10.6 bases as found previously¹.

The same value was obtained using micrococcal nuclease (not shown), but here the lengths of a proportion of the fragments produced is gradually reduced as the digestion proceeds. This is in contrast to the case of the two synthetic (A, T) polymers, where all fragments are progressively reduced in length (see below). We interpret the difference as due to the relative resistance to micrococcal nuclease⁵ of (G,C) stretches, compared with (A,T) stretches, present in random sequence DNA. The mechanism of trimming of DNA fragments has consequences for the production of nucleosome cores from

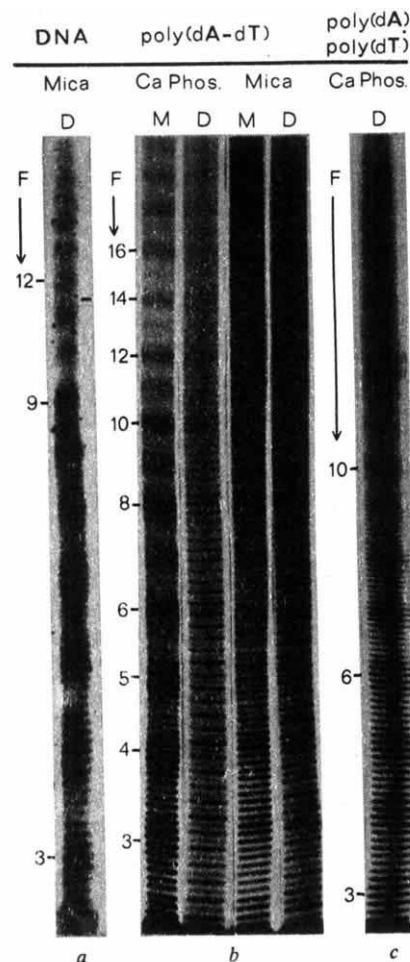
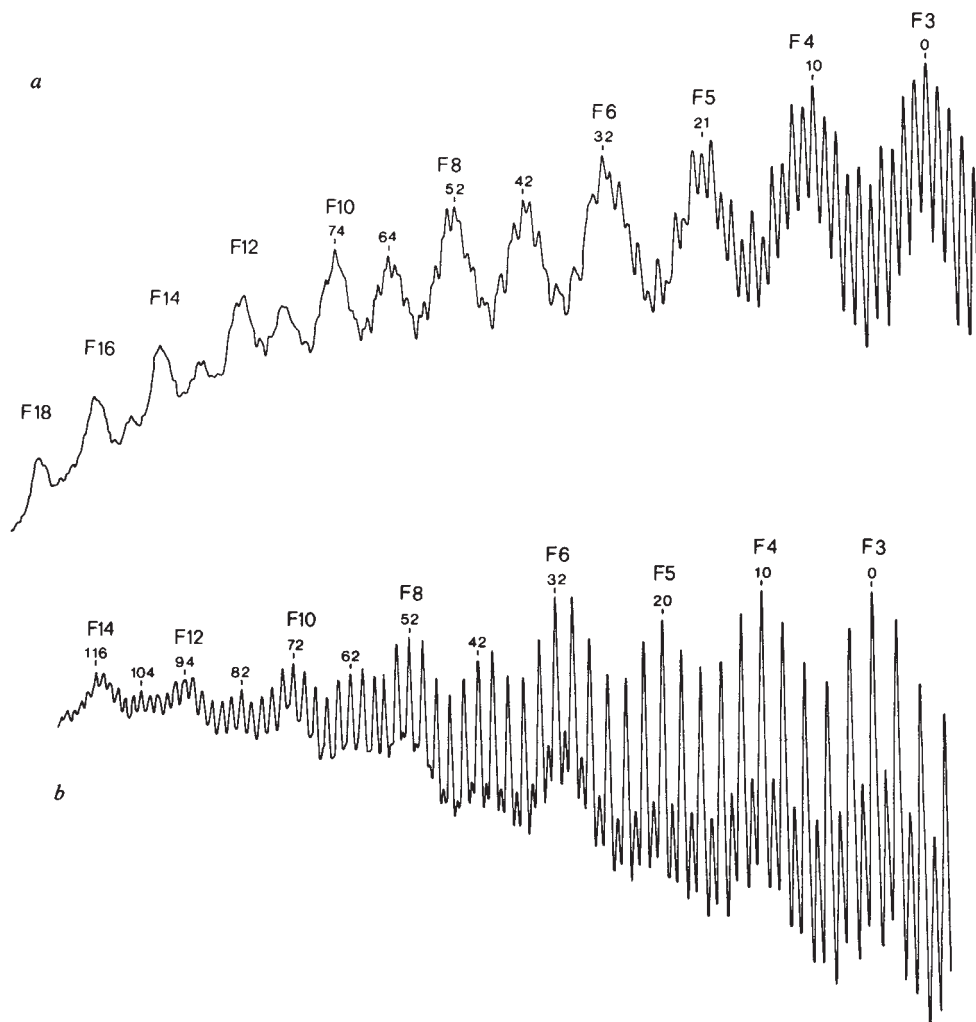


Fig. 1 Patterns of fragments produced by nuclease digestion of three DNAs immobilized on flat surfaces. After nick translation in the presence of [α -³²P]dATP (ref. 23), each DNA sample was extracted, precipitated and the DNA and poly(dA-dT) precipitates dissolved in 20 mM Tris pH 8.0 and 60 mM NaCl. Poly(dA).poly(dT) was dissolved in 20 mM Tris pH 8.0 only, because even low concentrations of salt tend to favour the formation of triple strand helices²⁴. Finally, the two synthetic polymers were annealed at 40 °C for 10 min and then stored at 5 °C. The purity of the synthetic DNAs was checked by nearest neighbour analysis. The calcium phosphate surface and the mica powder were prepared as described earlier¹, except that the concentration of the buffer in which the mica is resuspended has been halved, to reduce the destabilizing effect that Mg²⁺ ions have on the double helices of (A, T) polymers²⁵. *a*, Autoradiographs of random sequence DNA fragments from electrophoresis in a high-resolution denaturing gel made with 8% acrylamide and 1.35% methylenebisacrylamide. This system can resolve random sequence fragments differing in size by one nucleotide³. *b*, Poly(dA-dT); *c*, poly(dA).poly(dT) fragments from electrophoresis in denaturing gels made with 6% acrylamide and 0.3% methylenebisacrylamide, as used in DNA sequencing⁴. All nuclease digestions were carried out at 20 °C. D signifies DNase I and M signifies micrococcal nuclease. F stands for a set, or group, of fragments and the number indicates the size class, for example, F3 represents fragments of ~30 bases in length. The gel tracks shown in *b* have been assembled from the same gel, containing additional experiments not shown here.

Fig. 2 Densitometer tracings of the autoradiograph shown in Fig. 1*b*, (first two lanes); poly(dA-dT) digested with *a*, micrococcal nuclease; and *b*, DNase I. The fine peaks which represent poly(dA-dT) fragments differing in size by one nucleotide are numbered from the centre of band F3. In *a*, because of the base specificity of micrococcal nuclease, there is an alternation of intensity in the fine peaks and in *b*, because of the base specificity of DNase I (see text), fine peaks every two nucleotides dominate the pattern.



chromatin and will be discussed elsewhere (M. Cockell, D.R. and A.K., in preparation).

The results from a typical experiment on poly(dA-dT) immobilized on two different surfaces and digested with micrococcal nuclease or DNase I are shown in Fig. 1*b*. The intensity variations between successive fine bands is caused by the particular base specificities of the two nucleases used. Micrococcal nuclease cleaves poly(dA-dT) in solution at Ts with only a slight preference over As⁶ whereas DNase I cleaves almost exclusively at Ts, producing a large proportion of fragments differing in size by two nucleotides⁷ (Fig. 2*a,b*). The sets of fragments produced by micrococcal nuclease (lanes M) lie one or two nucleotides below the sets of fragments produced by DNase I (lanes D). This reduction in length depends on the extent of digestion, but all fragments seem to be reduced in length to an equal extent. Consequently, the distance between micrococcal nuclease cutting sites remains essentially constant.

The helical repeat of poly(dA-dT) bound to calcium phosphate, obtained directly from the densitometer tracings (Fig. 2*a,b*), is 10.5 bp with both nucleases. The intensity distribution in Fig. 1*b* (first two lanes) and Fig. 2*a,b* indicates that the helical periodicity is very close to 10.5 bp. There is an alternation in intensity between successive sets of intense fine bands, especially visible in the longer fragments, for example, F10 and F18. This shows that the repeat length seen by the enzyme is two helical turns corresponding to an exact integral number of base pairs ($10.5 \times 2 = 21$). Poly(dA-dT) bound to mica showed a slightly lower helical periodicity (Fig. 1*b*, last two lanes). The alternation of the intensities of the longer fragments is not so evident and the densitometer tracings (not shown) give a helical repeat of 10.4 bases.

Figure 1*c* shows the result of an experiment with the third polynucleotide, poly(dA).poly(dT), bound to calcium phosphate

and digested with DNase I. The densitometer tracing in Fig. 3 shows that the helical periodicity of poly(dA).poly(dT) is distinctly different from that of DNA or poly(dA-dT). The repeat length of the fragments produced is 10.0 bases. The periodicity observed using micrococcal nuclease is the same as that found with DNase I, but because the fragments are reduced in size very rapidly⁸ the measurements vary from site to site and are therefore less accurate (not shown). We were unable to obtain a ladder reflecting the double-helical screw from poly(dA).poly(dT) immobilized on mica. Presumably, when the polymer comes into contact with Mg²⁺ present in the mica suspension buffer, its double-helical nature is lost because of the propensity of poly(dA).poly(dT) to form triple-stranded helical structures⁹.

The enzyme digestion method for determining the helical periodicity of DNA is simple, direct and requires no corrections. The accuracy of the measurements of the distance between

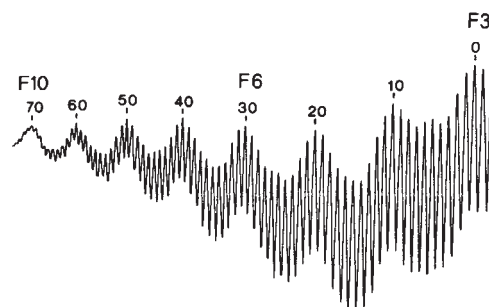


Fig. 3 Densitometer tracing of the autoradiograph shown in Fig. 1*c*. The fine peaks which represent poly(dA).poly(dT) fragments differing in size by one nucleotide are numbered from the centre of F3 to F10.

nuclease cutting sites depends mainly on the length of the largest DNA fragments that can be resolved to a resolution of a single nucleotide in the gel electrophoresis system, that is, on the number of helical repeats counted. The resolution of the homopolymer fragments is very good because the gel electrophoresis system used for sequencing DNA can be used⁴, but the random-sequence DNA fragments cannot be fractionated to the same resolution³. In our earlier paper on random-sequence DNA we mentioned that the changes caused by cation type and concentration and temperature on the screw of DNA^{10,11} would not be >0.1 bp per turn. In the present study with poly(dA-dT), where the precision of the results is higher, we find just such a change. The decrease in screw in the presence of millimolar Mg²⁺ is about 1% = 0.1 bp and is consistent both in sign and magnitude with previous work by others¹¹.

The results we have obtained demonstrate that the helical periodicity of DNA depends on the nucleotide sequence. Thus, the number of 10.0 bp per turn of the poly(dA).poly(dT) double helix in solution is distinctly different from the value of 10.4–10.6 we found for poly(dA-dT) and random-sequence DNA. These values are in good agreement with measurements by Wang who, using a completely different experimental approach from ours, has reported a value of 10.5 bp for DNA and 9.9 for poly(dA).poly(dT)^{12,13}.

It is not surprising that the same helical screw is found for random-sequence DNA and poly(dA-dT) because the former must contain, on average, equal numbers of purine and pyrimidines on each strand. The different helical periodicity observed for poly(dA).poly(dT) can perhaps be correlated with physicochemical studies which suggest that this polymer is different in some way from all other DNAs studied¹⁴. Poly(dA).poly(dT) fibres give essentially the same B-form X-ray diffraction pattern as other DNAs, but, unlike all other DNAs studied, no conditions have been found for its transition to a different form¹⁵. Furthermore, poly(dA).poly(dT) is the only DNA with a UV circular dichroism spectrum which does not fit a simple additivity rule accommodating many different DNA duplexes¹⁶.

The distinctly different helical screw we have found for poly(dA).poly(dT) could be of some biological significance. The previous finding that nucleosomes cannot be reconstituted from poly(dA).poly(dT), but are easily obtained from reconstitution with poly(dA-dT) or DNA^{17–19}, was previously explained by the high salt in the reconstitution system used, disproportionating poly(dA).poly(dT) into triple helical structures. However, the present results suggest an alternative explanation. There are also examples where the A+T-rich spacer regions between genes, such as the 5S genes of yeast^{20,21} and the histone genes of sea urchin²², contain long runs of As. Although these findings might not be coincidental, it is premature to conclude that such stretches of poly(dA).poly(dT) would exclude nucleosomes directly.

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In vivo consequences of plasmid topology

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The topology and physical chemistry of closed circular DNA molecules are well understood, but the significance for events within living cells is less well appreciated. It has been demonstrated recently^{1–3} that the torsional constraint which arises from negative supercoiling (that is, reduction of linkage) can induce localized novel secondary structure in isolated plasmid and phage DNA. Inverted repeats adopt hairpin-loop structures not found in relaxed DNA. This structural perturbation might be expected to have functional significance within the living cell, but clearly this requires that the torsional free energy be available for unhindered partition between alterations of twist and writhe. Microheterogeneity in DNA structure has recently attracted considerable interest, especially with regard to left-handed sections of duplex^{4–6}. The inverted repeats identified as sites of hairpin formation are relatively small, with stems of 13 base pairs (bp) or less. Whilst these hairpins could result in a relaxation of ~10% of the plasmid supercoiling energy, it was of considerable interest to try to construct stem-loop features about 10 times larger so as to study the topological consequences. In the cloning experiment described here, designed to produce direct or inverted 130-bp repeats depending on insertional orientation, no inverse species could be discovered, and deletion events were frequent. It is concluded that the inverted repeat deprives *Escherichia coli* of its antibiotic resistance. Cruciform adoption by the inverted species can totally relax the torsional constraint in the plasmid. These experiments highlight the importance of topological considerations in the genetics of closed circular DNA, and confirm the availability of torsional constraint *in vivo*.

Figure 1a shows the experimental basis of the investigation. A 130-bp *EcoRI*, *BstNI* fragment of pAT153 (ref. 7) was inserted back into the parent plasmid to generate either a direct repeat, or an inverted repeat with a 6-bp central loop, depending on orientation. Resulting colonies were screened by restriction enzyme analysis of plasmid DNA, using the predicted restriction maps shown in Fig. 1b. The most straightforward screening procedure involved *HindIII* digests of plasmid mini-preparations⁸ from each colony. As pAT153 has a single *HindIII* site at 29 bp (relative to *EcoRI* at 0 bp), the direct repeat recombinant should give a 130-bp fragment, and the corresponding fragment size from the indirect repeat molecule should be 70 bp. Figure 2a shows a typical restriction cleavage analysis of some recombinant plasmids. Analysis of 67 ampicillin-resistant colonies revealed that these comprised 21 recyclized parent plasmids, 25 direct repeat species, no inverted repeat species and 21 deletants. The most important comparison is that between direct and inverted repeats of 25/0. As the probability of insertion in either orientation is 50%, it is quite clear that selection at another level is operating in this experiment. A rather high level of deletants was observed (31%), several of which seem to be mutations which disrupt the symmetry of what would otherwise be inverted repeats. This again implies that it is the symmetry of the inverted repeat which is responsible for its non-viability. The high level of recyclized vector (31%) arises from the inherent low efficiency of blunt-end ligation reactions, coupled with the removal of inverse orientation events from the statistics. The statistics show clearly that an inverted repeat of unit length 136

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bp confers lethal properties on pAT153. This could arise by interference with the mechanism of plasmid replication or the expression of the ampicillin-resistance gene, such that resistance is no longer conferred on the host bacterium. The act of insertion *per se* is clearly not responsible for the effect, because the direct

repeats do not possess these properties, and thus we are left with the symmetry created by inverse insertion. However, the symmetry cannot be solely responsible for the lethal properties because smaller inverted repeats occur in ColE1 and pBR322^{1,2} and in recombinants derived from these plasmids³. The major

Fig. 1 a, Schematic outline of the construction of recombinant molecules derived by reinsertion of the 130-bp *EcoRI*, *BstNI* fragment of pAT153 (ref 7) into the flush-filled *EcoRI* site of pAT153, with a hexanucleotide asymmetric joining section. pAT153 was cleaved by *EcoRI* and the resulting 5' extensions 'filled in' using 2 units of the Klenow fragment of *E. coli* DNA polymerase I (Boehringer) in 50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mM β -mercaptoethanol with 0.25 mM of each of the four deoxynucleotides for 30 min at 15 °C. One-quarter of this was dephosphorylated using 20 μ g bacterial alkaline phosphatase (F grade, Worthington) in 20 mM Tris pH 7.5, 0.1% SDS for 30 min at 37 °C, for subsequent use as cloning vector. *EcoRI* linker (Collaborative Research) was 5' phosphorylated in two stages. 2.5 μ g of the double-stranded octanucleotide was incubated in 50 mM Tris pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol, 0.1 mM spermidine using 100 μ Ci of 5,000 Ci mmol⁻¹ [γ -³²P]ATP (Amersham) and 3 units of T4 kinase (New England Biolabs) at 37 °C for 60 min, followed by addition of unlabelled ATP to 1 mM and further kinase and incubation for an additional 60 min. The phosphorylated octanucleotide was ligated to the remainder of the flush-ended *EcoRI*-cleaved pAT153 by the blunt-end ligation procedure¹⁹, using 0.2 units of T4 DNA ligase (Bethesda Research) in 50 mM Tris pH 7.5, 10 mM MgCl₂, 20 mM dithiothreitol, 1 mM ATP for 2 h at 15 °C. After *EcoRI*, *BstNI* cleavage, the fragments were resolved on 5% polyacrylamide gel electrophoresis²⁰ and the 136-bp fragment removed by homogenization and extraction in 0.5 M NH₄-acetate, 10 mM Mg-acetate, 0.1% SDS, 0.1 mM EDTA²¹ at 37 °C, followed by binding to DEAE cellulose (Whatman), washing, elution and ethanol precipitation. After polymerase filling to give flush ends, this fragment was blunt-ended ligated into the flush-ended *EcoRI*-cleaved and dephosphorylated pAT153. After ligation, DNA samples were diluted to 100 μ l in 10 mM Tris pH 7.5, 10 mM CaCl₂ and 10 mM MgCl₂ and used to transform 200- μ l aliquots of CaCl₂-shocked *E. coli* K12 HB101 (ref. 22) as described previously²³. After 20 min at 37 °C in L broth containing 100 μ g ml⁻¹ carbenicillin (Pyopen), 200- μ l aliquots were plated on to agar containing 100 μ g ml⁻¹ carbenicillin and incubated at 37 °C for 16 h. (All restriction enzymes used in this study were from New England Biolabs or Bethesda Research Laboratories and used as directed.) Key: R, *EcoRI* site; H, *HindIII* sites; B, *BstNI* sites; BAP, dephosphorylation using bacterial alkaline phosphatase; PolI, flush-filling using DNA polymerase I (Klenow fragment); Ap^r, resistance to ampicillin. The rectangular boxes represent the synthetic *EcoRI* linker fragment, and the filled box represents the double-stranded AATTC unit. Filled triangles denote restriction enzyme sites, and open triangles denote restriction sites deleted during construction. b, Restriction maps of the *EcoRI* region of pAT153, and calculated maps for the direct and inverse recombinants, all derived from the sequence of pBR322 (ref. 24). Key: R, *EcoRI*; H, *HindIII*; B, *BstNI*; T, *TaqI*; H (smaller), *HaeIII*; h, *HhaI*. The thicker line denotes sequences between the *EcoRI* and *BstNI* sites of pAT153 which become duplicated in the recombinants. The filled boxes represent the joining hexanucleotide.

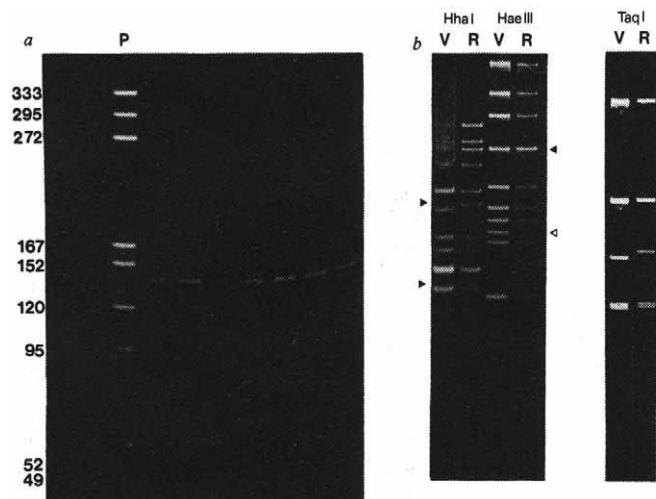
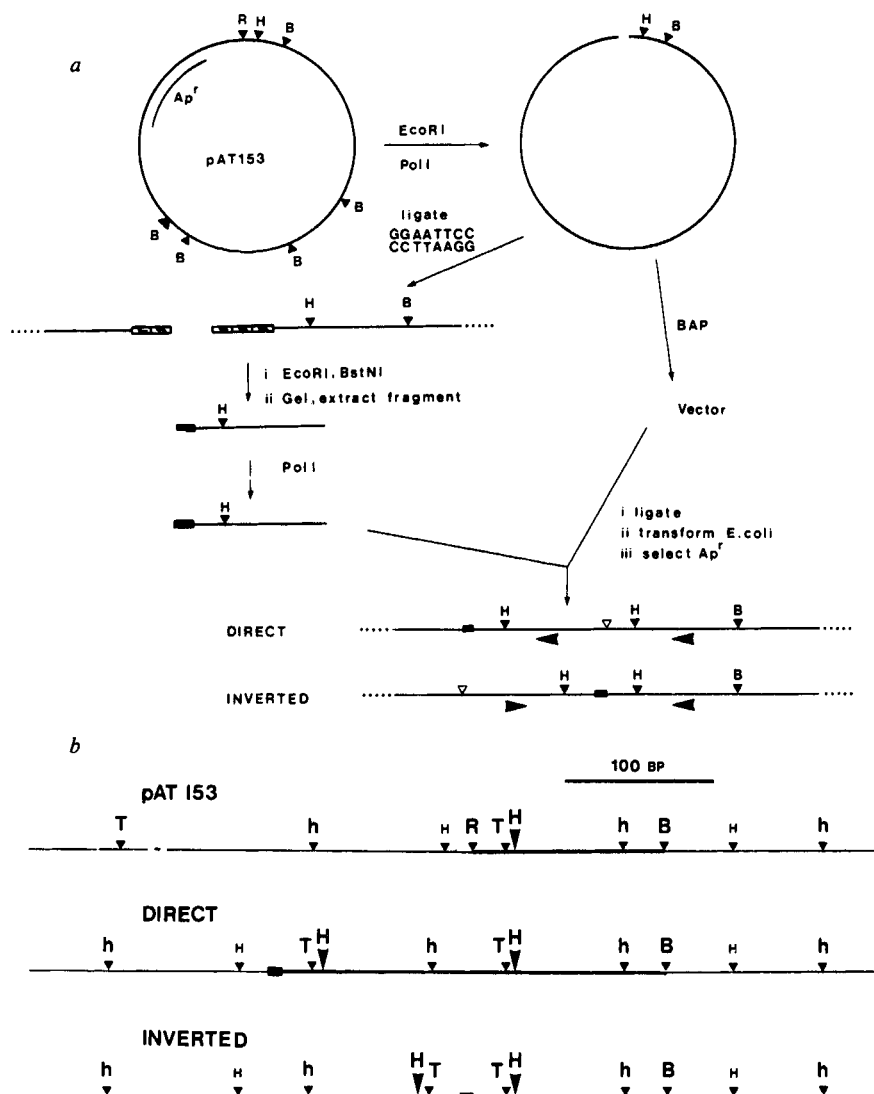


Fig. 2 Analysis of recombinants by restriction enzyme cleavage. a, *HindIII* cleavage of plasmid mini-preparations grown from individual colonies. The marker fragments (P) are *HaeIII*-digested phage PM2 DNA. Colonies were picked into 1 ml L broth solutions containing 100 μ g ml⁻¹ carbenicillin and grown at 37 °C for 16 h in the absence of chloramphenicol. DNA was prepared using a modification of the alkaline preparation method⁸ where all standing times were reduced to 5 min. After two ethanol precipitations and an optional phenol extraction, the DNA was sufficiently pure to be digested by restriction enzymes. This method gives a low chromosomal DNA background but to visualize small DNA fragments it was necessary to remove contaminating RNA by inclusion of 50 μ g ml⁻¹ RNase A (Sigma), preincubated at 95 °C for 10 min to remove potential DNase activity) in the restriction enzyme digestion. b, Detailed analysis of one clone containing a full-length 136-bp insertion. A 100-ml culture was grown from a 1-ml overnight incubation of one full-length recombinant, for 16 h at 37 °C in L broth containing 100 μ g ml⁻¹ carbenicillin but in the absence of chloramphenicol amplification. Plasmid DNA was purified from cell lysates by banding in caesium chloride²⁵. Key: V, pAT153 digests; R, recombinant digests. Solid triangles denote new (or double intensity) fragment bands; open triangles denote bands missing from the recombinant digests. Note that these digests unambiguously confirm the orientation of the inserted DNA, and the increase in length of the 206-bp *HhaI* and 368-bp *TaqI* fragments confirms the presence of the joining hexanucleotide.

difference between the inverted repeat created in this experiment, and those found in ColE1 and pBR322, is one of size. The ColE1 repeat has a unit size of 13 bp, whereas that in the potential pAT153 recombinant is 10-fold larger—130 bp.

I have observed previously¹ that small inverted repeats present in supercoiled circular DNA are centrally hypersensitive to S_1 nuclease, implying the adoption of hairpin-loop structure. On forming a cruciform type of structure an alteration of twist occurs for the molecule, given by

$$\Delta Tw = n/10.4 \quad (1)$$

where n is the total number of base pairs involved in the formation of the hairpin. (The number of 10.4 is derived from an estimate of the twist of solution DNA being 10.4 bp per helical turn⁹.) The parameters which describe the topological properties of supercoiled DNA are related by the expression^{10,11}

$$\Delta Lk = \Delta Tw + Wr \quad (2)$$

where ΔLk is the alteration in linkage from the most relaxed topoisomeric conformation, and Wr is the writhing number. The linking number for pAT153 has been measured as 18 ± 1 (Fig. 3), which equation (2) shows must be partitioned between alteration of twist or writhe, either of which is energetically unfavourable. However, the twist alteration produced by cruciform formation allows the rest of the molecule to 'relax' by the extent

$$R = \frac{-n}{10.4 \Delta Lk} \times 100\% \quad (3)$$

thus, for ColE1 ($n = 30$ and $\Delta Lk \approx -27$)¹² a relaxation of twist and writhe of 11% should occur. For the inverse recombinant pAT153 species relaxation becomes 142% ($n = 266$), that is, the molecule can be totally relaxed. This, then, is the likely basis of the lethal properties of these large inverted repeats.

Further examples of selection against inverted symmetric units can be found in the literature^{13,14}, and another example has recently been noted in this laboratory (W. Tacon, unpublished results), when it was found that only tandem repeats of a 111-bp fragment could be obtained in a multiple-copy cloning experiment. One explanation previously proposed¹⁴ was that the symmetry might facilitate strand switching during replication. However, there is no *a priori* reason that such a process should occur only in longer inverted repeats. It seems more likely,

therefore, that the topological effects are the basis of these observations. Another instance of such properties was noted by Gellert *et al.*¹⁵, who were unable to propagate a circular dimer of the longer *EcoRI*, *BamHI* fragment of pBR322. Another example may be found in a study of post-transformational rearrangement in modified pSM19035 (ref. 16), where manipulations designed to generate a large perfect inverted repeat resulted in spontaneous deletion events which removed the centre of symmetry.

Several consequences follow from these observations. First, the strong selection against inverted repeats demonstrates that topological effects are operative on DNA within the living bacterium, that is, that the torsional constraint arising from linkage reduction by gyrase is 'available', rather than being locked up in writhing around histone-like proteins. This energy is available to drive processes requiring strand separation, such as transcription^{17,18}, and possibly the alteration of DNA secondary structure¹⁻³. It is therefore perhaps not surprising that the (possibly transient) release of this free energy into cruciform structures has deleterious consequences. Second, the present study demonstrates that inverted repeats can strongly influence the functional properties of supercoiled molecules. The difference between the stable direct species and the unstable inverted species constructed in these experiments is quite subtle—the reversal of polarity of 130 bp in a 3.7-kbp molecule at a region distinct from both ampicillin-resistance and replication functions. Yet this modification in some way deprives the cell of its resistance to the antibiotic. Finally, these studies have practical significance, for they imply that the topological consequences of certain cloning operations should be considered. Thus, 'head to head' dimers of fragments above a limiting size are likely to be impossible to clone. Furthermore, plasmid 'libraries' may be incomplete if inverted repeats are present in the original nucleic acid cloned.

The size limitations on cloning ability are imprecise at present, but some limits may be set. The stability of cruciform structures will be proportional to stem length, but inversely proportional to loop size^{1,3}, whereas the topological consequences depend on the size of the whole feature. Stem sizes of 9–13 bp do not result in plasmid instability¹, whilst we have noted in this laboratory that 30-bp duplications ($R = 32\%$) cannot be cloned inverted in pAT153. However, longer inverted repeats have been propagated where the loop size is 12 nucleotides. As I have noted previously³, the symmetry requirements for cruciform structure formation are stringent.

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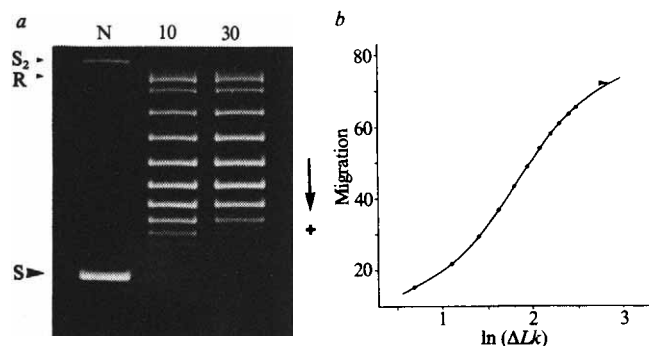


Fig. 3 Measurement of the relative linking number (ΔLk) of pAT153 by gel electrophoretic resolution of partial topoisomerizations^{26,27}. *a*, 1.2% agarose gel electrophoresis (room temperature) of native supercoiled and partially relaxed pAT153. 0.8 μ g of pAT153 was incubated with 2 units of *Agrobacterium tumefaciens* topoisomerase (Bethesda Research) at 37 °C in 20 mM Tris pH 7.8, 2 mM $MgCl_2$, 7 mM β -mercaptoethanol for the times indicated (min), followed by electrophoresis at 50 V for 16 h in 40 mM Tris pH 8.0, 5 mM Na-acetate, 1 mM EDTA²⁸. Key: N, fully supercoiled pAT153; S, R and S₂, positions of migration of fully supercoiled monomer, nicked monomer and supercoiled dimer species. *b*, Extrapolation of migration of topoisomers to that of the native supercoiled pAT153 (shown by the arrow) indicates that $\Delta Lk = -18$. Migration of individual topoisomers was measured by microdensitometer scanning of photographic negatives. The lower bands were assigned by comparison of identical partial topoisomerizations on agarose gels run at room temperature and 5 °C (ref. 16). Thus, the most slowly resolved topoisomer in *a* was assigned to $\Delta Lk = -2$. A small error (estimated to be ± 1) may result from the extrapolation procedure.

Sequence homologies between tonin, nerve growth factor γ -subunit, epidermal growth factor-binding protein and serine proteases

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Tonin¹, a protein isolated from rat submaxillary gland, has interesting proteolytic activities. It is able to liberate angiotensin II from angiotensinogen (or a shorter synthetic tetradecapeptide corresponding to the first 14 residues) and from angiotensin I; it is also able to release peptides from β -lipotropin (β -LPH), corticotropin (ACTH) and their common precursor pro-opiomelanocortin (POMC) leaving intact the β -endorphin and the Met-enkephalin entities^{2,3}. Here, we describe the sequencing of 95 of the 272 amino acids of tonin. Our results reveal extensive sequence homology of tonin with the γ -subunit of nerve growth factor (NGF) and epidermal growth factor (EGF)-binding protein (both of which also have proteolytic activities), and also with serine proteases.

Tonin is a single polypeptide chain of 272 amino acids having a molecular weight of 28,700; sequence analysis has revealed isoleucine and proline at the NH₂ and COOH termini respectively⁴. We have now extended the NH₂-terminal sequence up to 65 cycles with excellent repetitive yield while identifying blanks and uncertain residues in the previously reported sequence, as shown in Fig. 1a. In addition, we identified 30 other residues in a cyanogen bromide fragment, giving the definitive sequence of 35% of the molecule (Fig. 1b). While this work was being carried out, the sequence of the γ -subunit of mouse NGF became available⁵, making it possible to compare the amino acid sequences.

As indicated in Fig. 2a, alignment of the first 65 residues of the γ -subunit and tonin reveals only 15 differences—77% homology. Furthermore, the sequence Val-Leu-Thr-Ala-Ala-His-Cys, which is found in all serine proteases except haptoglobin, a recently proposed serine protease homologue⁶, is present in rat tonin; it does contain a conservative substitution, namely the isoleucine at position 35 in place of the common leucine residue. This segment is very important because it contains the histidine residue typical of the active site of all serine proteases. The cysteine residue at position 24 is part of the disulphide loop maintaining the proper orientation of the histidine residue. The relationship between rat tonin and serine proteases can also be seen by comparison of the first 65 residues with known sequences: the homology with rat tonin is highest for pancreatic enzyme kallikrein⁷ (55%) and trypsin⁷ (48%), with other serine proteases slightly less homologous⁶⁻¹³.

Comparison of the NH₂-terminal sequence of a cyanogen bromide fragment of tonin with positions 95–124 of the γ -subunit (Figs 1b and 2b) also reveals 21 identical positions out of 30, thus showing a striking similarity (70%) between rat tonin, γ -subunit and serine proteases.

Although the present results reveal only a partial (35%), but definitive, sequence of rat tonin, it is nevertheless clear that tonin isolated from rat submaxillary gland is very closely related to the γ -subunit of mouse NGF and also probably to the EGF-binding protein. Our data do not show conclusively that tonin represents a variant of the γ -subunit of the rat NGF because NGF could not be detected in the submaxillary gland of the rat^{14,15}; however, this possibility cannot be ruled out. Also interesting is the fact that the isolated tonin is a single poly-

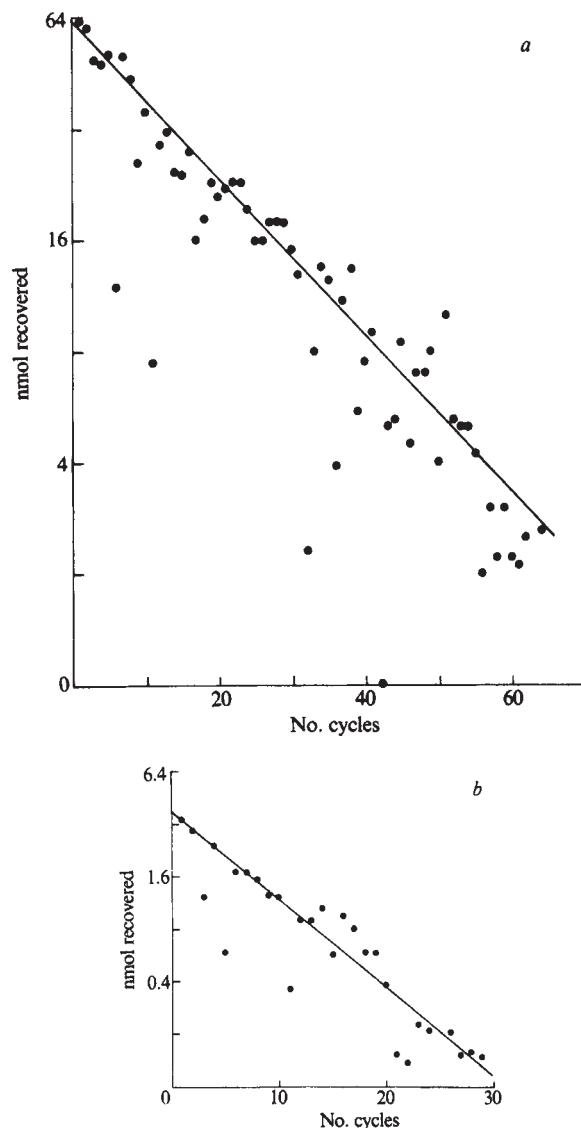


Fig. 1 a, Automatic NH₂-terminal degradation of reduced and alkylated rat tonin. Sequencing was carried out on 2.6 mg (87 nmol) of protein using 3 mg of polybrene precycled seven times and a 0.3M Quadrol programme on an updated Beckman 890B sequenator fitted with a cold trap. Phenylthiohydantoin (PTH) derivatives were obtained after conversion in methanol/HCl using an in-line sequemat P6 autoconverter. Separation and quantification were carried out by HPLC using an ultrasphere-ODS column and a tetrahydrofuran acetonitrile gradient. Quantitative yields of PTH-amino acids corrected for background and normalized to PTH-norleucine internal standard are illustrated as a function of residue number. The slope and intercept were obtained by a linear regression analysis on selected stable PTH-amino acids. Repetitive yield thus obtained was 95.2% while the carry-over never exceeded 1% except for Glu and Asp residues which are retained by the polybrene. b, Automatic NH₂-terminal degradation of a reduced and alkylated (using ³H-iodoacetic acid) fragment of rat tonin obtained by cyanogen bromide cleavage. Sequencing was carried out on ~10–15 nmol using the conditions described above; the repetitive yield for that sequence was 89%.

peptide chain comprising 272 amino acids, whereas the γ -subunit, as reported, is composed of 233 amino acids distributed in what seems to be two or three chains linked by disulphide bridges. This observation supports Thomas *et al.*'s proposal⁵ that a limited, perhaps autocatalytic, proteolysis of a single-chain form of γ -subunit generates a two- or three-chain form. Rat tonin is clearly related to the serine proteases family, especially to trypsinogen, kallikrein and plasminogen. On the other hand, the substrate specificity of this enzyme seems to be

* Deceased.

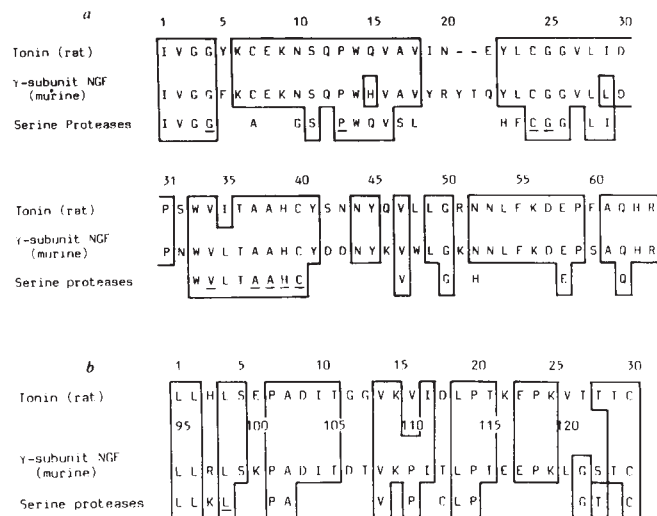


Fig. 2 *a*, Comparison of the amino acid sequence (in the single-letter code, ref. 16) of the NH₂-terminal portion of rat tonin with corresponding segment of γ -subunit of mouse NGF as determined by Thomas *et al.*⁵ and with conserved segments of serine proteases according to Dayhoff⁷. The underlined residues correspond to invariant position in the known sequence of all serine proteases. *b*, Comparison of the amino acid sequence of a cyanogen bromide fragment of rat tonin with residues 95–124 of γ -subunit of mouse NGF and conserved segments of serine proteases.

quite different from the arginine-specific activity of NGF γ -subunit and EGF-binding protein because it is able to cleave after specific arginine residues and after suitably located lysine, phenylalanine, tyrosine and tryptophan residues depending on the pH used for the digest^{2,3}. For example, at pH 5.0 it can selectively cleave the pro-opiomelanocortin precursor to yield intact ACTH involving an arginine and phenylalanine cleavage at the NH₂ and COOH termini respectively. Thus, this enzyme expresses a selective chymotryptic-tryptic-type activity. The digestion studies clearly indicate the importance of the conformation of the substrate in directing the site of cleavage^{2,3}. Originally, it was proposed¹ that tonin might be involved in the local generation of angiotensin II in tissue; its newly found similarity with NGF γ -subunit and EGF-binding protein suggests other possible roles for this enzyme and its substrate specificity.

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Methionyl-tRNA synthetase shows the nucleotide binding fold observed in dehydrogenases

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A striking common structural feature has emerged from the comparison of the X-ray crystallographic studies of several dehydrogenases. In lactate dehydrogenase¹, soluble malate dehydrogenase², alcohol dehydrogenase³ and glyceraldehyde-3-phosphate dehydrogenase⁴ similar foldings have been described in the region which binds the coenzyme NAD, whereas no significant similarities were observed in the chemical sequences. The occurrence of a characteristic 'nucleotide binding fold' (the so-called Rossmann fold) has also been observed in horse muscle phosphoglycerate kinase⁵, in phosphorylase^{6,7} and, with some topological deviations, in other kinases^{8–11} as well as in the flavin-binding domain of flavodoxin¹². If one assumes that these structural homologies are the result of a divergent evolutionary process, it is then tempting to predict a similar pattern of structure-function relationship in other nucleotide-binding proteins, in particular in aminoacyl-tRNA synthetases. We show here that methionyl-tRNA synthetase does show the same nucleotide binding fold.

Native *Escherichia coli* methionyl-tRNA synthetase, a dimer of molecular weight (MW) 172,000, has never been obtained in a crystalline form. However, a fully active monomeric fragment which can be prepared by a controlled proteolysis¹³, crystallizes in space-group P2₁ with $a = 78.1$ Å, $b = 46.2$ Å, $c = 87.9$ Å, $\beta = 108.8^\circ$. The crystals contain one molecule of MW 64,000 per asymmetric unit¹⁴. The structure of this active fragment has been determined at 2.5 Å resolution by the multiple isomorphous replacement (MIR) method using five heavy-atom derivatives. The diffracted intensities were recorded photographically with an oscillation camera and measured using a rotating drum densitometer (Centre de Microdensitométrie, Orsay). Some statistical data on the quality of the measurements and the heavy-atom derivatives are given in Table 1. The MIR phases were improved by a solvent modification technique similar to that used by Schevitz *et al.*¹⁵ in the study of yeast tRNA^{Met}. The resulting electron density map was easily interpreted for most of the molecule, except in one small region situated in the bottom right part of Fig. 1. A detailed account of the experimental procedures and a complete description of the

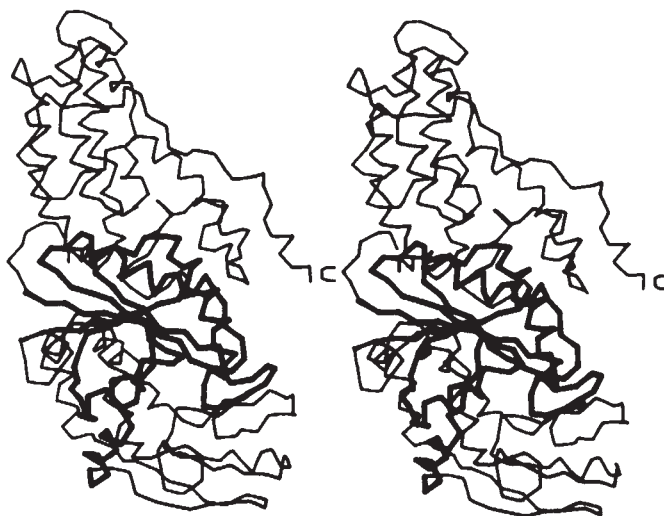


Fig. 1 Stereoscopic drawing of the α -carbon backbone of methionyl-tRNA synthetase based on unrefined coordinates measured from the model. The central part (thick line) corresponds to the nucleotide binding domain. The molecule is viewed down the crystallographic b axis.

structure will be given elsewhere. An atomic model (scale 1.25 cm per Å) was built, which accounts for 480 amino acid residues.

There is no complete chemical sequence available for methionyl-tRNA synthetase but its molecular backbone is

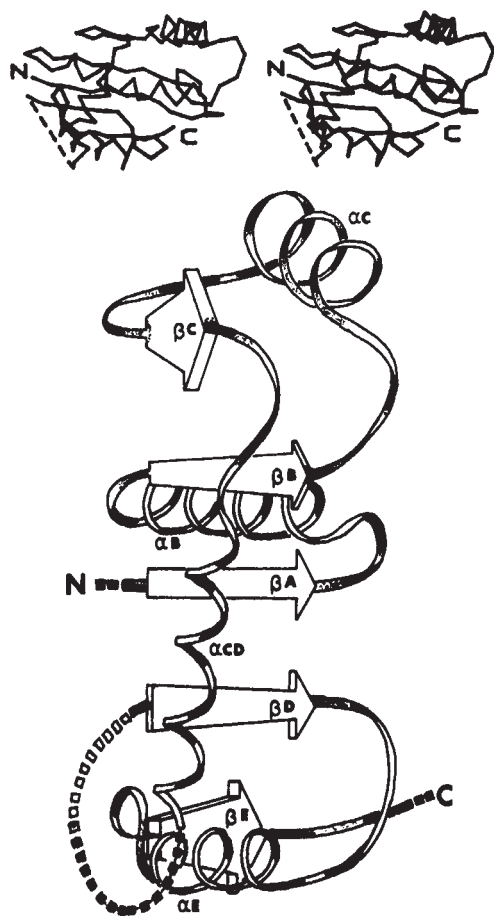


Fig. 2 A stereo view of the nucleotide binding domain in methionyl-tRNA synthetase, together with a schematic representation of its structural elements. The nomenclature of the helices and β strands are those proposed by Hill *et al.*². The β strands are represented by arrows pointing towards their C-terminus. The dotted chain between helix α CD and strand β D represents that part of the molecule which is depicted at the bottom of Fig. 1 (thin line). The view is approximately down the a^* axis. N and C represent the amino and carboxyl ends of the chain, respectively.

shown in Fig. 1. The elongated molecule ($90 \times 52 \times 44$ Å) consists of three distinct domains: (1) a central core (thick line in Fig. 1) formed by five parallel strands separated by helices and containing the N-terminus; (2) a less regular structure (thin line at the bottom of Fig. 1) containing ~135 amino acid residues, which can be visualized as an intrusion between the third and fourth strands of the central core; and (3) a third domain (thin line, top of Fig. 1) rich in helices, which bears the C-terminus of the polypeptide chain.

The most salient feature of the structure is, by far, the central core, which shows the topological characteristics of a 'nucleotide binding domain': (1) it is composed of a five-stranded parallel pleated sheet with helices connecting the strands. (2) The sequential order of the strands, as shown in Fig. 2 is, from top to bottom, β C- β B- β A- β D- β E. (3) Helices α B and α C are 'below' the pleated sheet (Fig. 2) while helices α CD and α E are 'above' it. (4) The nucleotide 8-bromo-ATP, which was used as a heavy-atom marker, binds near the C-terminal end of the pleated sheet (in our interpretation this binding involves contacts with the loop between strand β A and helix α B and with side-chains of helix α B). These features clearly indicate a remarkable structural homology with the nucleotide binding domain of dehydrogenases. Note that although the structure of tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* seemed at first to bear no structural relationship to other proteins¹⁶, an improved model revealed the existence of a Rossmann fold around the ATP binding site¹⁷.

Methionyl-tRNA synthetase is thus a new member of the family of proteins which possess the characteristic Rossmann fold. This supports the hypothesis that many nucleotide binding proteins may derive from a common ancestral gene^{18,19}. By gene fusion and duplication, this gene would have evolved in a divergent evolutionary process to give rise to the modern dehydrogenases and aminoacyl-tRNA synthetases (the situation is less clear for kinases which show a rather wide variety of topologies in their ATP-binding domain). The nucleotide binding domain of methionyl-tRNA synthetase, however, has a unique feature, that is, the helix α CD and the strand β D are far apart in the sequence (they are separated by ~135 residues). These residues build a domain (bottom of Fig. 1) whose structure bears little resemblance to a Rossmann fold. This extra domain could be the result of the insertion of a segment of DNA into the ancestral gene²⁰. Conversely, aminoacyl-tRNA synthetases could mirror the existence of a longer ancestral gene which, after deletions, would now code for the nucleotide binding domains in the dehydrogenases. Interestingly, the presence of an extra domain has also been reported for pyruvate kinase (PK)¹¹ as compared with triose phosphate isomerase (TIM)²¹. The two enzymes show a characteristic barrel of eight

Table 1 Quality of intensity measurements and heavy-atom derivatives

	∞ -7.3	7.3-5.5	5.5-4.4	4.4-3.7	3.7-3.2	3.2-2.8	2.8-2.5	Overall
<i>N</i>	713	942	1,544	2,209	2,961	3,621	4,020	16,010
R_{sym} (native)	0.015	0.022	0.025	0.027	0.037	0.060	0.078	0.040
R_{sym} (Pt)	0.020	0.025	0.026	0.030	0.040	0.060	0.073	0.041
R_{sym} (U)	0.018	0.022	0.024	0.026	0.034	0.051	0.063	0.035
R_{sym} (Sm)	0.021	0.026	0.027	0.030	0.038	0.054	0.062	0.039
R_{sym} (Pt+U)	0.034	0.038	0.037	0.040	0.052	0.073	0.10	0.053
R_{sym} (8-Br-ATP)	0.029	0.033	0.037	0.040	0.049	0.071	0.089	0.052
<i>P</i> (Pt)	2.26	2.02	1.72	1.38	1.39	1.30	1.17	1.44
<i>P</i> (U)	2.30	2.12	1.65	1.54	1.44	1.34	1.17	1.46
<i>P</i> (Sm)	2.07	2.32	1.69	1.59	1.56	1.68	1.50	1.66
<i>P</i> (Pt+U)	2.13	2.55	1.69	1.58	1.56	1.49	1.28	1.59
<i>P</i> (8-Br-ATP)	1.10	1.08	0.84					0.97
<i>m</i>	0.75	0.73	0.70	0.68	0.66	0.64	0.57	0.65

Statistical data on the quality of intensity measurements and heavy-atom derivatives, given as a function of resolution. *N* is the number of phased reflections. Due to the geometry of the oscillation method, about half of the reflections are measured more than once; the R_{sym} factor ($R_{\text{sym}} = \sum | \langle F_i \rangle - F_i | / \sum F_i$) gives a measure of the agreement between symmetry-related reflections. *P* is the phasing power of the heavy-atom derivatives, that is, the ratio of the r.m.s. heavy-atom scattering to the r.m.s. lack of closure: $P = [\sum f_h^2 / n]^{1/2} / [\sum (F_{\text{ph}}^{\text{obs}} - F_{\text{ph}}^{\text{calc}})^2 / n]^{1/2}$. The derivatives used were obtained with $\text{K}_2\text{Pt}(\text{CN})_4$, $\text{K}_3\text{UO}_2\text{F}_5$, $\text{Sm}(\text{NO}_3)_3$ and 8-bromo-ATP (8-Br-ATP). The last derivative was used only at 4 Å resolution. *m*, Figure of merit. For each derivative (including the parent) ~20% of the measured reflections were rejected (too weak or poor R_{sym}).

parallel strands connected by helices which is continuous in TIM, and in PK contains ~100 additional residues, inserted between the third strand and the third helix.

Knowledge of the complete amino acid sequence for methionyl-tRNA synthetase may hopefully provide further insight into its taxonomic relationships with other nucleotide-binding proteins.

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Cationic control of O₂ affinity in lugworm erythrocrucorin

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The erythrocrucorins (extracellular haemoglobins from annelids¹) have molecular weights of 3.4×10^6 , contain 60–192 O₂-binding haem moieties per molecule and are much more complex than the tetrameric vertebrate haemoglobins^{1–5}. However, they perform the same function, carrying O₂ from the respiratory surfaces to the tissues, and exhibit similar cooperativity in O₂ binding and inhibitory heterotropic interactions between O₂- and proton-binding sites (Bohr effects), although these functions show greater adaptive variation than in the vertebrate pigments^{2,3}. Whereas erythrocrucorin–O₂ affinity is insensitive to the anionic organic phosphate cofactors like glycerate-2,3-bisphosphate and ATP^{1–3}, which depress the O₂ affinity of vertebrate haemoglobin inside the red blood cells, it is increased by inorganic salts^{4–6}. This effect is important physiologically because annelids lack significant capacity for osmotic regulation and experience large fluctuations in blood electrolyte levels^{8,9}. I show here that, in contrast to the situation in man, where anionic cofactors and protons decrease haemoglobin–O₂ affinity by specifically depressing the O₂ association equilibrium constant of the pigment in the deoxygenated state^{10–13}, inorganic cations govern the O₂ affinity of erythrocrucorin from the burrowing, intertidal lugworm, *Arenicola marina*, by preferentially modifying the association constant of the (almost fully) oxygenated form. In contrast to the vertebrate mechanism, which optimizes O₂ unloading in the tissues, this alternative control mechanism in erythrocrucorin seems to be adaptive to O₂ loading at the low O₂ tensions generally characteristic of the microenvironments of erythrocrucorin-bearing annelids^{1–3}.

Figure 1 shows that NaCl increases O₂ affinity and cooperativity (measured as $n_{\max}$, the maximum slopes of the Hill plots) of *Arenicola* erythrocrucorin by increasing the association constant for binding the last O₂ molecules, without significantly affecting that for binding the first molecules to the deoxygenated pigment. These constants, which are respectively referred to as K_R and K_T , by analogy with the R and T states of vertebrate haemoglobin, without implying the existence of only two discrete conformational states, are estimated by lines drawn asymptotic to the experimental curves at extreme high and low O₂ saturations (see Fig. 1). Divalent cations exert greater effect than monovalent ones at the same anion concentration and also slightly increase K_T (Fig. 1). These results imply preferential cation binding to erythrocrucorin in the oxygenated form and provide a rare example of an heterotropic effector that increases O₂ affinity in haem pigments.

The differential effects of salts on K_T and K_R raise the free energy of haem–haem interaction, ΔG (which equals $RT \ln (K_R/K_T)$ where R is the gas constant and T the absolute temperature¹⁴). At pH 7.37, which approximates the *in vivo* value at 15 °C (refs 1, 2), the K_R/K_T ratio indicates a 33-fold higher affinity for the last compared with the first O₂ molecules bound, and a ΔG value near 8.4 kJ mol^{–1} in the absence of salts. The presence of 0.6 M Na⁺ or 0.12 M Mg²⁺ (which approximate the blood levels in lugworms from normal seawater¹²) raises K_R/K_T to ~68 and ΔG to ~10.0 kJ mol^{–1} (Fig. 1, right panel). The mechanism of ionic interaction in erythrocrucorin is radically different from that in human haemoglobin, where glycerate-2,3-bisphosphate lowers K_T (reducing the association constants for the first three but not the last O₂ molecule) and where neutral salt acts in a qualitatively similar manner except that it also slightly increases K_R (refs 10–12). Both control mechanisms, however, have the same net effects, increasing ΔG and $n_{\max}$.

Proton concentration also acts mainly on K_R of erythrocrucorin but in the opposite direction to salt concentrations, shifting the oxy-asymptote to lower affinities and reducing ΔG and $n_{\max}$ (Fig. 2). This also contrasts with human haemoglobin, where lower pH reduces K_T and only slightly affects K_R , raising ΔG and cooperativity¹³. The present data allow estimation of the Bohr effects for binding of the first and last O₂ molecules ($\Delta \log K_T/\Delta \text{pH}$ and $\Delta \log K_R/\Delta \text{pH}$, respectively) as –0.20 and –0.74, showing that most protons are released on binding of the last O₂ molecules.

The shifts in the oxy-asymptotes of *Arenicola* erythrocrucorin imply the existence of different R states whose O₂ affinities depend on cation and proton binding. Ions thus affect not only the apparent equilibrium constant for the transition between the deoxy- and oxy-forms but also the non-exclusive binding coefficient $c (=K_T/K_R)$, in contradiction of the two-state model¹⁵ as defined for human haemoglobin, where c is invariant of the concentration of the allosteric effector.

The fact that the ion sensitivity of erythrocrucorin resides with K_R , rather than with K_T as in human haemoglobin, seems to be adaptive to O₂ transport in hypoxic (O₂-poor) conditions in the natural habitat of annelid worms. In contrast to air-breathing animals where loading O₂ tensions generally are sufficient to saturate the pigment fully, so that O₂ transport can only be improved by increasing O₂ unloading, O₂ transport in hypoxic microenvironments depends on O₂ loading and thus K_R . In animals like *Arenicola*, a salt-induced increase in K_R and thus in $n_{\max}$ and O₂ affinity will favour oxygenation of the erythrocrucorin in the gills during tidal emersion, when severe burrow hypoxia¹⁶ coincides with high blood salt levels as a result of saline, dense, high-tide water remaining in the burrows⁸. Again, the proton sensitivity of K_R , resulting in a greater Bohr effect at high degrees of O₂ saturation, will augment the O₂ affinity difference between blood at relatively high O₂ tension and pH (as in the gills) and blood at lower O₂ tension and pH (as in tissues)¹⁷. As the published data show that in nature tissue O₂ tensions in *Arenicola marina* fall sufficiently to unload the pigment at low tide¹⁶ and that gill–tissue pH differences do occur¹⁸, the salt and

Fig. 1 Effects of inorganic salts on O_2 equilibria of *Arenicola* erythrocytes, measured in 0.05 M Tris-HCl buffer at 15°C and pH 7.37; haem concentration is 0.17 mM; $\bar{Y}$ is the fractional O_2 saturation. Left: Hill plots in the absence of cation (Δ) and in the presence of 0.12 M NaCl ($\circ$), 0.25 M NaCl ($\bullet$), 0.62 M NaCl ($\square$) and 0.12 M $MgCl_2$ ($\blacklozenge$). Right: effects of NaCl ($\bullet$) and $MgCl_2$ ($\blacklozenge$) on the association equilibrium constant for the first and last O_2 molecules bound (K_T and K_R , respectively) and on the free energy of haem-haem interaction, ΔG . The erythrocytes, collected as described previously, were stripped of ions by passage through mixed ion-exchange resin (Amberlite MB-3) and dialysis against distilled water. Cation concentrations were varied by the addition of solutions of analytical grade cation-chloride salts and controlled by chloride measurement using a Radiometer CMT-10 titrator. The O_2 equilibria were measured with a diffusion chamber technique^{19,20} except that the stepwise increases in O_2 tensions were generated by cascaded gas mixing pumps (Wösthoff) while absorbance changes between zero and full saturation (when equilibrated with pure N_2 and O_2 , respectively) were monitored on recorders with high sensitivity (Radiometer REA 112) units. Extremely low and high saturations were measured by amplifying absorbance increments near zero and full O_2 saturation in separate runs, observing previously described precautions¹⁴. The asymptotes were fitted by eye. With the upper asymptotes the saturation points were additionally estimated from extrapolations in plots of $\Delta \bar{Y}$ against $1/p_{O_2}$ (ref. 13). The application of small amounts of erythrocyte solutions (layer thickness $\sim 10 \mu m$) minimized equilibration time and meterythrocyte formation, which was always less than 1% and corrected for graphically²⁰.

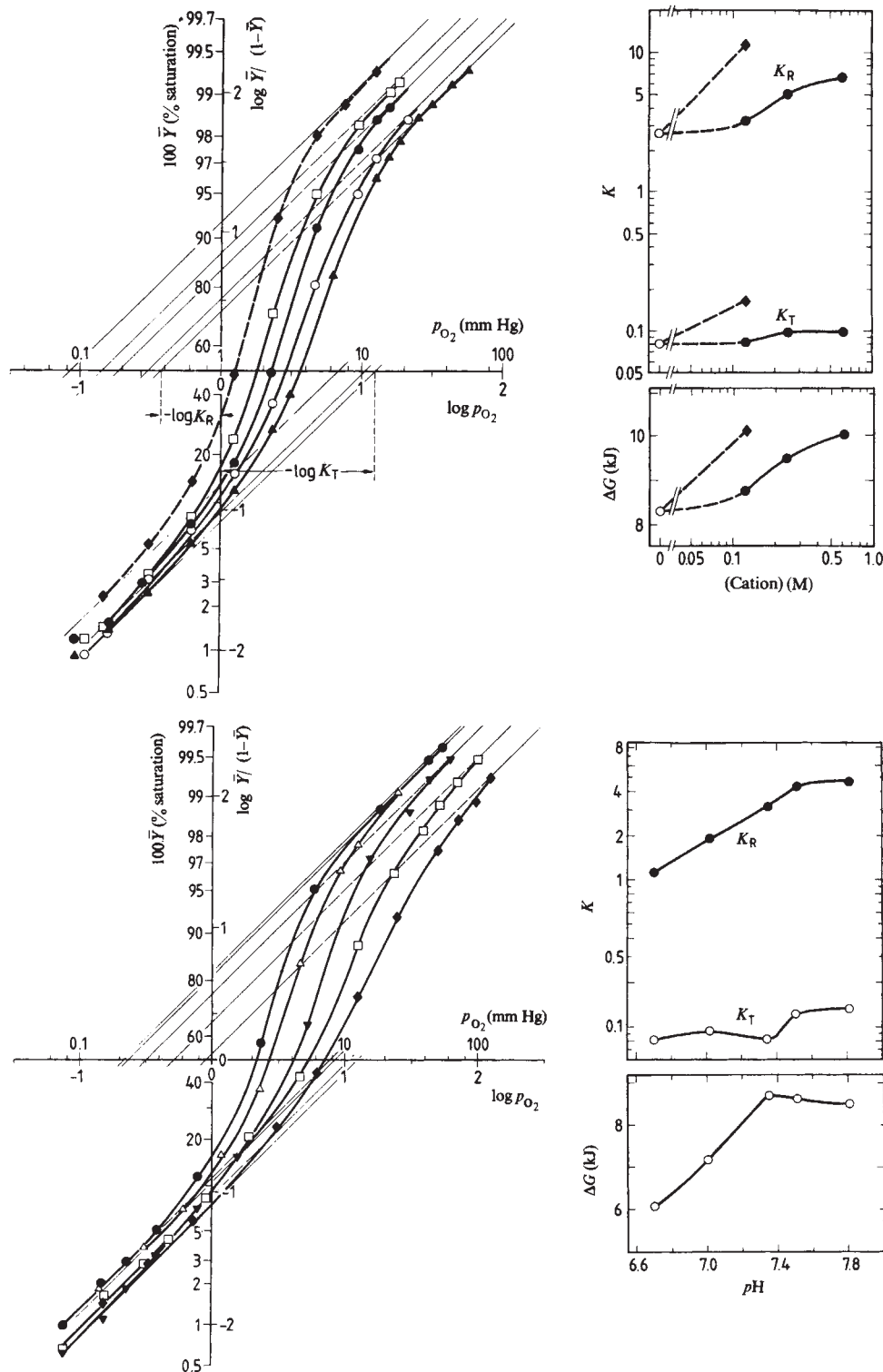


Fig. 2 Effects of pH on O_2 equilibria of *Arenicola* erythrocytes, measured in 0.05 M HEPES-NaOH buffer at 15°C. pH values (left panel): 7.81 ($\bullet$), 7.51 (Δ), 7.35 (∇), 7.02 ($\square$) and 6.70 ($\blacklozenge$). The correspondence of curves in the presence of HEPES and Tris buffers (compare ∇ with Δ in Fig. 1) indicates the absence of significant buffer effects. Other details are as for Fig. 1.

proton interactions will increase the capacity of the blood for transporting O_2 .

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MATTERS ARISING

Rebuttal of criticisms of remote viewing experiments

MARKS¹ has argued that our first two remote viewing (RV) studies (with subjects Price and Hammid)² should be discounted as evidence for the RV hypothesis. He claims that sensory cues in the subject-generated RV transcripts provide sufficient information to permit judges to blind-match transcripts to target sites on an artefactual basis. The evidence, however, does not support this claim.

First, with regard to the Hammid series. When one examines the details of Marks' criticism (see ref. 3) one finds that the criticism about cueing is based on an error-in-fact on the part of Marks; he erroneously assumed that the target list given to judges was in the order of target usage, that is, unrandomized. That is incorrect. That list, which Marks used to formulate his criticism, was randomized by random number generator to prevent the very possibilities he raises. When this fact is taken into account, one finds that his criticism is voided at its foundation. Specifically, his criticism is derived from the fact that the transcripts were dated; however, the dates do not provide the useful cue information posited by Marks—they would have done so only under the (incorrect) assumptions about target order made by Marks. As a result, Marks' attempt to associate the transcripts to the target sites on the basis of the alleged cues failed.

With regard to the Price series. While not questioning the data collection procedures or commenting on the near-photographic accuracy of some of the individual descriptions, Marks and Kammann hypothesized in their first critique that the successful judging of the Price series might be due to sensory cues in the transcripts that provide information as to, for example, whether a given trial was early or late in the series⁴. In response to this we arranged to have the series independently rejudged, using transcripts from which the suggested cues had been removed. The rejudging resulted in the same seven out of nine target/transcript matchings as in the original study, indicating that it was the quality of the transcripts themselves, rather than the presence of cues, that was responsible for the successful outcome². This rejudging is rejected by Marks because of the potential confounding factor of the publication of the original study.

This leads us to a second re-analysis of the Price series for which, in order to give the Marks' hypothesis its best chance, we assume all potential cues remain and are used to maximum advantage; we then rigorously assess the consequences. In the strongest case for cues the probability of a correct match can at most be increased from one-ninth to one-third; in another

case from one-ninth to one-seventh; and in two other cases from one-ninth to one-eighth. The remaining five cases gain no direct advantage from cues, just indirect advantage from not using as possibilities the ones already used in the more advantageous cases. When one takes into account the resulting constraints due to cues, the number of possible target/transcript matchings is reduced from $9! = 362,880$ to 68,760 combinations, by exact count. Assuming that the cues are used to maximum advantage, we find that the significance level associated with obtaining at least seven matches in nine trials (as was done) in a forced-choice, non-independent assignment of transcripts to target sites (the most conservative statistic) is only reduced from $P = 10^{-4}$ to $P = 3.9 \times 10^{-4}$, still a quite significant result. (A paper containing the details of this analysis has been published elsewhere⁵.) Thus, the Marks hypothesis that the presence of extraneous cues accounts for the significance of the Price series result receives no support.

We would summarize by first indicating that we recognize that our original RV studies are not without flaw, and the potential effect of possible cues deserves critical examination. However, in our continuing re-examination of this work we find that the suggested cueing artefacts, although providing some potential for confounding, are yet well below the magnitude necessary to account for the strength of the results. As a result, this re-examination of the data has provided yet additional support for the hypothesis indicating the existence of a human remote sensing capability. This conclusion is supported by the continuing replication of RV experiments in our own and other laboratories^{6,7} for which the Marks concerns no longer apply due to the increased sophistication of the procedures in use.

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Expression of herpesvirus-induced antigens in human cervical cancer

DREESMAN *ET AL.*¹ have described the expression of two herpes simplex virus type 2 (HSV-2) antigens, designated ICSP

11/12 and ICSP 34/35, in 38% of cervical tissues with pathological findings of severe dysplasia or carcinoma. Their undocumented statement that previous studies used antisera against HSV virions and gave rise to equivocal results is inaccurate and generates controversy where none exists. Indeed, the expression of HSV-2 antigens in cervical anaplastic tissue has been consistently and unequivocally demonstrated using antisera prepared against extracts of HSV-2-infected cells that contain both structural and non-structural viral proteins. To our knowledge, there is no report describing the failure to identify HSV-2 antigens in cervical anaplastic tissue when such have been studied. Had the authors' comment on the equivocal nature of previous results been valid, their data demonstrating the presence of two antigens, the viral identity of which may be questionable to some critics, in only 38% of patients with anaplastic findings could hardly support their conclusion that HSV-2 is a factor in human cervical cancer¹.

Using antisera against total viral antigens, HSV-2 antigens were demonstrated by immunodiffusion and crossed immunoelectrophoresis in extracts of cervical tumour cells^{2–4}. Similarly prepared antisera were used by Athanasiu *et al.*⁵ in immunofluorescent staining of frozen sections of cervical tumour biopsies. Expression of HSV-2 antigens was described in 8 of 24 (33.3%) specimens from cervical carcinoma, a percentage remarkably similar to that described by Dreesman *et al.*¹, using similar specimens. The observation that viral antigens are preferentially expressed in cells on the peripheral part of the carcinoma mass towards the vagina and/or cervical canal^{6,7} has led some investigators to conclude that the proportion of reactive patients could be greatly increased by studying the cervical cells that have naturally exfoliated or have been induced to exfoliate artificially. Immunofluorescent staining of such specimens with antisera against total viral antigens in six independent studies^{6–11} has demonstrated the expression of HSV-2 antigens in cells of 61–81.2% of patients with dysplasia and 92–100% of those with invasive cancer or carcinoma *in situ* (CIS). This compares with a reactivity of 0–9.4% in normal women (Table 1).

We showed previously that antisera against an antigenic fraction designated AG-e, that consists of two viral proteins (ICP 12, ICP 14)¹², stains cervical anaplastic cells from patients with dysplasia, CIS or invasive cancer, but not from normal women¹⁰. Consistent with this finding, antisera against ICP 12 or ICP 14 also stain the cells from patients with these pathological findings¹¹. It is significant that ICP 12 shares a number of

Table 1 % Of patients exfoliated cells staining with antisera to viral antigens*

Diagnosis	Total†	AG-e‡	ICP 12	ICP 14
Normal (N)	0–9.4	0	0	0
Dysplasia (D)	61–81.2	69–72.6	57	51
CIS and invasive (Inv)	91.6–100	91.4	100	75

* Athanasiu *et al.*⁵: 33.3% of frozen sections of cervical carcinoma stained with antiserum to total viral antigens. Normal tissue was negative.

† Pacsa *et al.*⁸: N = 9.4%, D = 61%, CIS/Inv = 94%. Adelsi *et al.*⁹: N = 0, Inv = 100%.

Minhui *et al.*⁶: N = 9%, Inv = 100%. Aurelian *et al.*^{7,10}: N = 0, D = 81.2%, CIS/Inv = 91.6%.

‡ Smith *et al.*¹¹: N = 0, D = 72.6%, CIS/Inv = 91.4%. Aurelian *et al.*¹⁰: N = 0, D = 67.6%, CIS/Inv = 86.4%.

properties with Dreesman's ICSP 11/12—the two proteins have the same molecular weight and both bind DNA^{1,13}. Antisera against them stain the nuclei of HSV-2-infected cells, but staining of cervical anaplastic cells is cytoplasmic and perinuclear^{1,11}.

We suggest that the findings of Dreesman and his colleagues cannot be considered in isolation of previous evidence in this field. They neither offer conceptually new information, nor do they help elucidate a hitherto equivocal or controversial issue. The significance of their findings lies specifically in their confirmatory nature. Only in this context can the authors' conclusion be accepted, that HSV-2 is a factor in cervical carcinoma.

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POWELL ET AL. REPLY—We have no intention of generating controversy as Smith, Gupta and Aurelian have suggested. Our aim was to examine sections of cervical tissue biopsy specimens for the presence of two specific HSV antigens. We detected both proteins in about one-third of the samples of abnormal tissues¹. As we detected the HSV antigens in the unstressed biopsy specimens, this result clearly differs from that of Aurelian *et al.*², in their paper "HSV-2 antigens absent from biopsied cervical tumor cells: a model consistent with latency" wherein

they were unable to demonstrate the presence of HSV-2 antigens in cervical tumour biopsies from 29 patients. We would agree that all published reports have found at times expression of HSV antigens in exfoliated cervical cells.

Previous results are equivocal. Thus whilst Athanasiu *et al.*³ reported the presence of virus antigens in cervical tumour biopsy specimens using antisera to crude virus antigens and the indirect immunofluorescence test, this result was not obtained by Nahmias *et al.*⁴, Aurelian *et al.*² or by ourselves¹. Even in studies of exfoliated cells, reports differ as to the presence of virus antigens in cells from normal patients or from patients with bland disorders and in the proportion of patients with overt disease showing positive reactions with antisera to crude virus protein mixtures^{2,5,6}. It is important not to gloss over such differences as they may be highly significant.

Our approach was quite different from that of previous reports. We selected two proteins ICSP 11, 12 and ICSP 34, 35 because they are both DNA-binding proteins. Both are known to be virus specific and have been mapped using intertypic recombinants to specific regions of the viral genome. They were purified to homogeneity and only then used to prepare antisera. The sera were tested to ensure that they only reacted with the polypeptide used for immunization. These reagents obviously differ considerably from the crude or partially purified antigen preparations used by other groups to prepare antisera for previous studies^{2,4–6}. With these reagents two independent studies have detected expression of HSV antigens in about one-third of cervical tumour biopsies^{1,7}. We were totally unable to detect HSV antigens in the same specimens using potent general antisera to total virus proteins, in contrast to results reported by Aurelian's group with exfoliated cervical cells (60–90% of dysplastic or CIS/invasive cancer patients samples positive even using antisera to crude infected cell extracts).

Finally, we are amazed at the casual way in which Smith, Gupta and Aurelian associate their ICP 12 and ICP 14 (which they have clearly shown to be structural proteins capable of inducing neutralizing antibody⁸) with ICSP 11, 12 which is a non-structural protein totally absent from

even the worst preparations of purified virus. Our reagents and our results are clearly different from those of Aurelian and her co-workers.

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Recent thrusting in the Appalachians

STUDIES of incremental strain have been used in an attempt to understand recent tectonism in regions, such as eastern North America, which are generally considered to be tectonically inactive. Yet one of the best pieces of evidence of recent tectonism and incremental strain is seismicity, and eastern North America has historically been seismically quite active¹.

Schäfer² has studied apparent recent tectonism in the Appalachians using offset drill holes to indicate incremental strain. Incremental strain is observed in drill holes following construction of presplit highway cuts in the Cumberland–Allegheny Plateau, and in the western Valley and Ridge of Tennessee, West Virginia and Pennsylvania.

We have examined three of the four localities described by Schäfer, and other localities nearby. Our additional data suggest that tectonic stresses resulting in faulting are responsible for the observed offsets but that there is no evidence of post-palaeozoic tectonism.

Examination of exposures along Interstate Highways 40 and 75 in Tennessee and at Oak Flat, West Virginia, revealed numerous offset drill holes in Pennsylvanian and Devonian sandstone (and sandstone/shale). All observations were confined to near-vertical presplit roadcuts in which the rocks are minimally weathered providing less chance of error caused by measurements on loose blocks.

In all offset holes containing multiple offsets, or in any succession of offsets from

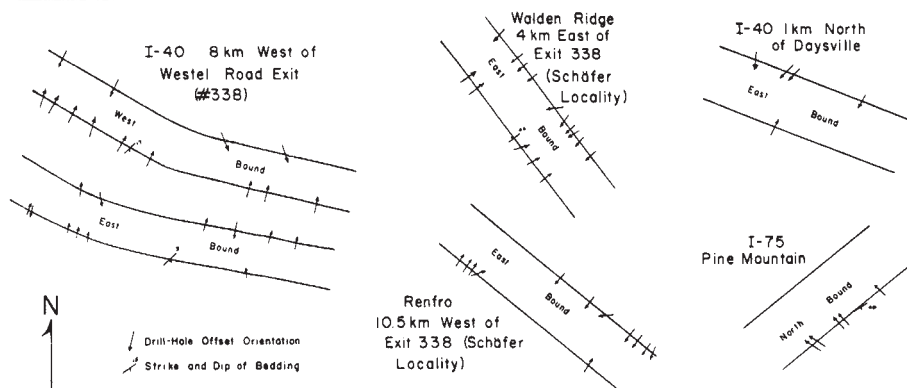


Fig. 1 Orientations of displacement for drill holes at the localities in Tennessee.

the base to the top of a cut in the Tennessee localities, the amount of offset increases upwards. Also, in most drill holes in which there are multiple offsets, the offset vectors are nearly parallel. Where offset vectors are not parallel, differences in motion may generally be explained by relative rotation of one of the upper blocks during blasting, later slumping, readjustment across major joints, or some other local process. At the West Virginia locality south-west of Oak Flat, the sense of movement is reversed from the base to the top of the cut on the north side of the highway. On a lower-to intermediate-level fracture the drill holes are offset away from the highway, whereas on an upper-level fracture the offset vectors have moved towards the highway.

A consistent relationship between offset vectors and highway cut orientation was noted. Orientation vectors are consistently oriented approximately normal to highway cuts. Most vectors symmetrically converge towards the centre of the highway (Fig. 1). At one locality in Tennessee, measurements were made in two parallel cuts of westbound and eastbound lanes separated by 30–50 m of rock. Offset vectors converge symmetrically towards the centre of the westbound lane, but vectors on both the north and south sides of the eastbound lane point north (Fig. 1). Using an elastic rebound mechanism, this pattern may be explained by excavation of the westbound lane first, initiating rebound and release of elastic strain energy throughout the mass. The differential in total or average offset of drill holes from the westbound to the eastbound lane (consistently greater effects in the westbound lane) favours a difference in time of excavation for the two lanes.

Offsets may cease to exist from one side of prominent joints (properly oriented) to another. One side of a joint may be offset as much as 30 cm whereas drill holes on the other side of the joint may be offset only 0 to <2 cm.

Stresses which produce observed strains, such as offset drill holes, may be simply interpreted as having a tectonic origin². However, relatively high near-surface *in situ* stresses may also originate from non-tectonic processes³.

Static loads derived from the weight of rock material composing a topographic high may become resolved into shearing stresses directed along properly oriented existing planar discontinuities, such as subhorizontal bedding. As long as these stresses are less than the shearing strengths of the rocks involved or less than the stresses necessary to reactivate existing planar discontinuities, no permanent strain would result. There would also be some dependence of storage of elastic strain energy on the elastic moduli of the rock mass. The most favourable orientations for static load-derived shearing stresses to be relieved would obviously be those which would permit movement outwards from the source of stress. A downward-directed static load may be relieved effectively by horizontal motion, provided this otherwise normal stress may be transformed into shearing stresses (Fig. 2). Existing subhorizontal planar discontinuities (bedding or fractures) may provide shear surfaces along which the stresses may be relieved. Motion along these surfaces may be accelerated during blasting where rapidly expanding gases are forced along bedding or fracture surfaces. The gases separate the rock mass and effectively lubricate it to overcome the coefficient of internal friction along the surfaces, so that accumulated elastic strain energy is released. Further strain may be caused by subsequent creep along discontinuities.

Blocks which are surrounded by properly oriented joints may contain no stored energy. Joints generally dip 70° or more. Those which are sufficiently open (not locked) and are oriented at low to moderate angles to the outward-directed shearing stresses may be relieved without offsetting drill holes.

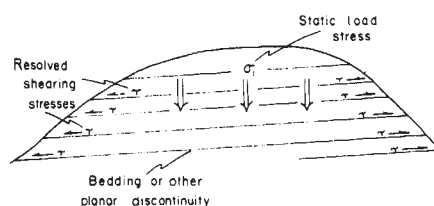


Fig. 2 Possible mechanism for producing subhorizontal shearing stresses from a nontectonic source.

We conclude that offset drill holes are a common phenomenon in the Cumberland-Allegheny Plateau of Tennessee and West Virginia where bedding is nearly horizontal. Drill holes are offset by release of stored elastic strain energy produced by static load. Offset drill holes in the Cumberland-Allegheny Plateau and in the Valley and Ridge are not related to recent tectonism. All tectonic structures present here predate highway construction and are probably Palaeozoic structures.

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SCHÄFER REPLIES—Hatcher and Webb have observed additional offsets of drill-holes with vectors oriented normal or oblique to the highway cuts. I agree that many of these displacements result from the release of static load-derived strain which is a common feature in the Appalachians and in other mountainous areas.

Hatcher and Webb stated that I simply interpreted those offsets as having a tectonic origin. However, I did not consider any offset vector which was directed towards or away from a highway, although a tectonic component could also be involved. Offset vectors oriented parallel to the highway cuts were not included, either, in my analysis when the excavation of the road was directed radially to a topographic high. As of sites 1 and 2 at Interstate highway 40, Tennessee, which is NW-SE-oriented, a topographic high is located north-east of the highway. Also at the West Virginia site the highway cut runs tangentially to a topographic high in the north-west. Thus, it is not surprising that Hatcher and Webb observed numerous drillholes with offset vectors normal or oblique to the road cuts. Other non-tectonic offset vectors may even run in the direction of the road-cut when static load-derived tangential tensile stresses induce dip-slip movements.

The borehole offsets which I considered, however, are all parallel (in one case sub-parallel) to the highway cuts revealing considerable rock masses to be upthrust by tectonic forces and not by blasting. The tectonic origin of these faults evidently is not doubted by Hatcher and Webb as they did not mention any of these borehole offsets in their discussion.

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BOOK REVIEWS

Time to change

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THIS book deserves to be read, and discussed, at several different levels, and not all levels can be covered in a single short review. The chemist or physicist who wishes to understand Prigogine's concept of "dissipative structure", and its application to systems far from thermodynamic equilibrium, for which he received the Nobel prize for chemistry in 1977, will find in Part II — "The Physics of Becoming" — a clear explanation of where both equilibrium and linear non-equilibrium thermodynamics become inadequate, and of how an externally imposed inhomogeneity, such as a temperature gradient, produces a new kind of order. Examples are given from physics (convection in the atmosphere), chemistry (periodic chemical reactions) and biology (cell accretion).

As its title implies, the book is more than that. Prigogine's Nobel award citation referred to his "bridging the gap between biological and social fields of enquiry", and he sets out here to share his insights with a wider reading public. His central character is Time, and he states the problem in his preface: to relate three different concepts of time occurring in dynamics (time as a simple parameter with no preferred direction), thermodynamics (time has direction, but leads inescapably towards "heat death") and biology (time as evolution and, ultimately, history).

In the preface, Prigogine acknowledges a debt to past giants in the field, Ludwig Boltzmann and Jacques Monod. In this work and its companion volume (*La Nouvelle Alliance*, written jointly with Isabelle Stengers and published by Gallimard in 1979), he makes a strong bid to join them.

But he is no philosophical soulmate of these two. Both describe themselves unhesitatingly as materialist in outlook, and Prigogine recounts how Monod rebuffed his attempt to establish common ground, classifying his programme for unifying the physical and social worlds as a variety of animism. Possibly this somewhat intemperate rejection led Prigogine, in his turn, to misunderstand Monod. For he quotes the passage from *Chance and Necessity* about man being "a stranger in the world from which he evolved by accident" as though Monod was referring to life as the strange feature. A more careful reading reveals that the strange feature, peculiar to our species, is *consciousness* — that which leads us

From Being to Becoming: Time and Complexity in the Physical Sciences. By Ilya Prigogine. Pp.272. ISBN hbk 0-7167-1107-9; ISBN pbk 0-7167-1108-7. (W. H. Freeman: 1980.) Hbk \$24.95, £16.70; pbk \$12.95, £7.95.

ultimately to scientific and philosophical enquiry about our environment. So if, as Prigogine proposes, we need a "second time" to describe irreversible processes, including life, how can he be sure that we do not need a "third time" to describe consciousness?

It is to Boltzmann, however, that Prigogine mainly addresses this work. He begins by noting, like Boltzmann and Gibbs before him, that in a purely dynamical system, based on a hamiltonian evolution function, it is not possible to construct a state function having the time-directional property of entropy. The "Poincaré-Misra theorem" (Chapter 7) simply puts this long-known result into the modern terminology of Lyapunov functions. Yet every thermodynamic system has an entropy. Ask a physical chemist and he will measure it for you. Hence the problem.

Gibbs's solution, which Prigogine rejects, was to say that the irreversibility of thermodynamic processes is an illusion, a creation of the physical chemist's imagination. Prigogine quotes some fragments of the Einstein-Besso correspondence which suggest that Einstein also held this view. But a closer examination of those letters, especially the passages dealing with Brownian motion, shows that, in fact, Einstein supported Boltzmann.

Boltzmann's solution was to propose that, in addition to the dynamical laws of evolution of a large system, we should require the initial state to be one of "molecular chaos". He anticipated Prigogine's diagram 7.3 and asserted that, since a state with all the molecular velocities reversed does not have this property, it will almost never occur in any actual physical system.

Prigogine, applying some new results obtained by mathematicians working in ergodic theory, thinks he has *derived* Boltzmann's molecular chaos from the dynamics. But has he? I believe not.

That is not to deny that he and his co-workers have achieved a great deal. They have shown, more clearly than most, that Boltzmann's definition of chaos has to be extended to include higher order correlations in the motions of neighbouring

molecules. And, for certain simple time-evolving systems, of which the most discussed is the non-dynamical Baker's transformation, they show how to construct an entropy operator which is compatible with the evolution operator. But the very fact that they end up with an entropy *operator* shows that they, like Boltzmann, are considering not a single microstate, but a set of states on which only certain averages are specified. To specify a probability measure on this set is to impose a more sophisticated molecular chaos hypothesis. So Boltzmann, rather than Prigogine, invented the concept of "second time".

In his remarks on Einstein's refusal to accept quantum mechanics, Prigogine is really out of his depth. It is the case that some quantum theorists are now proposing to abandon the 400-year-old notion of space going from Galileo through Einstein, on which all science since the Renaissance is based, preferring a "biological" space in which a change in one place produces instantaneous changes everywhere else. Such a feature, called "non-locality", seems to be an unavoidable consequence of quantum theory, and Einstein drew our attention to it nearly 50 years ago. But, in his enthusiasm for biological models, based on such phenomena as the cooperative motion of the parts of a chicken embryo, Prigogine thinks he sees common ground with the elementary particle "zoologists" in their high-energy physics laboratories. This is unwise, because the non-locality some physicists speak of has never been observed, and quite possibly it never will be. On the other hand, the non-locality exhibited by a chicken embryo certainly does not require superluminal signals to sustain it. It is easily subsumed within a materialist world-picture of the type supported by Boltzmann and Einstein. This is especially the case once full account is taken of the field concept, something which Prigogine almost totally ignores — Faraday does not rate a mention, and Maxwell enters only through his contribution to kinetic theory.

This is not a great book, and Prigogine has not yet achieved what Boltzmann did in *Populäre Schriften* or Einstein in *The World As I See It*. But it is a very good book, and the ideas contained within it promise better things to come. □

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What's new in the mechanisms of enzyme regulation?

Edwin G. Krebs

Protein Phosphorylation in Regulation: Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation. Vol. 1 of *Molecular Aspects of Cellular Regulation*. Edited by P. Cohen. Pp.273. ISBN 0-444-80226-6. (Elsevier/North-Holland Biomedical: 1980.) \$63.50, Dfl. 130.

As the title of this first volume in a series on cellular regulation suggests, the emphasis is on new systems that for the most part have not been reviewed extensively heretofore. The editor of the series, Philip Cohen, who is one of the world's foremost authorities on protein phosphorylation, provides an introductory chapter that gives an excellent summary of the well-established role of this process in the regulation of glycogen metabolism. His succinct account will provide readers with a broad outline of the history and evolution of the general principles of protein phosphorylation, but at the same time will not burden them with an enormous load of information that is available elsewhere. A more exhaustive treatment of glycogen metabolism in this book would have detracted from the reviews that follow. These include chapters by different authors on pyruvate kinase, acetyl CoA carboxylase, hydroxymethylglutaryl CoA reductase, steroidogenesis, triacylglycerol synthesis, muscle contraction, protein synthesis, the phosphorylation of ribosomal proteins and the phosphorylation of histone H1. Parenthetically, it should be noted that the word "enzymes" is used in a very loose sense in this volume, in that the topics covered include the phosphorylation of proteins ordinarily not thought of as enzymes.

The individual contributions, which, with one exception, are clear and well written, follow a general theme developed in the introductory chapter. Brief descriptions of the more classical enzymology of a given system, including non-covalent regulation, are followed by the more recent work concerned with phosphorylation-dephosphorylation. The latter, which constitutes the principal thrust of each article, is generally handled by progressing from *in vitro* phosphorylation to the more difficult *in vivo* situations. Most of the authors make an effort to apply criteria of physiological relevance to the phosphorylation phenomena that have been observed. This aspect is especially well handled in sections on the regulation of pyruvate kinase, the control of protein synthesis and the modification of histone H1. A final chapter in the book, again written by Cohen, highlights common features of the various phosphorylation systems and forecasts future directions that will be taken by workers in this field; in addition,

he introduces an interesting hypothesis concerning the mechanism of action of insulin in the regulation of protein phosphorylation.

The book can be recommended for readers who desire timely, concise reviews of work on various aspects of metabolic regulation controlled by protein kinases and phosphoprotein phosphatases. It is

designed more for the non-specialist than for the specialist, but anyone reading the volume will profit from the experience, particularly if he reads it in its entirety.

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Newtonian style and scientific revolution

Richard S. Westfall

The Newtonian Revolution, with Illustrations of the Transformation of Scientific Ideas. By I.B. Cohen. Pp.414. ISBN 0-521-22964-2. (Cambridge University Press: 1981.) £18, \$37.50.

TWENTY-five years ago, Professor Cohen's influential *Franklin and Newton* (American Philosophical Society, 1956) established him as the foremost student of Newtonian natural philosophy. Over the intervening years a constant stream of publications has at once expanded our understanding of Newton and the scientific revolution and maintained Cohen's position. To that stream he now adds another major volume, *The Newtonian Revolution*, the revised text of the Wiles Lectures delivered at the Queen's University of Belfast. As the two-part title suggests, the book divides into two major sections, one on the Newtonian revolution in science, the other on the doctrine of transformations as an interpretive explanation of the development of scientific ideas. Though the book contains several lengthy asides which are connected only tenuously to the central argument, the two sections maintain a fundamental unity by focusing on the same developments in Newtonian science. In Cohen's view — a view that most historians of science would enthusiastically endorse — the elaboration of the *Principia* was the single most important step forward in the course of modern science. The appearance of Professor Cohen's detailed analysis of it constitutes a major event in the historiography of science.

While the title of Part I recalls Thomas Kuhn's *Structure of Scientific Revolutions*, it is Part II, *Transformations of Scientific Ideas*, which speaks directly to the issues Kuhn raised. Where Kuhn argued for discontinuities in the growth of science, revolutions characterized by sudden switches in the perception of nature, Cohen stresses continuity in which every revolution is achieved by the transformation of received concepts. He is presently at work on a general exposition of

his theory of scientific growth. In the present volume he illustrates it by examining the same events that are central to his understanding of the Newtonian revolution. For the latter he develops the important concept of the Newtonian style by which he seeks both to identify the essence of Newton's contribution and to characterize the nature of modern science. Central to the Newtonian style is the use of mathematical constructs which successive modifications make ever more adequate to the full complexity of physical reality. Equally important is the postponement of enquiry into causes until maximum agreement between the construct and the world of experience has been attained. The idea of the Newtonian style enables Cohen to distinguish Newton's approach from that of other quantifiers and geometrizers in seventeenth century science. No one insisted more on the role of geometry in natural philosophy than Kepler, but, as Cohen shows, Kepler started with causal hypotheses and arrived at mathematical constructs, whereas the Newtonian style proceeded in the opposite order. The primacy of causal hypotheses likewise separated Newton from his continental critics, who were not inconsiderable mathematicians. As I indicated, the concept of a Newtonian style in science, which Cohen argues persuasively was a more important ingredient in his enduring influence than the specific content of the *Principia*, strikes me as a major contribution to our growing understanding of the scientific revolution.

I feel bound to add that, in my opinion, there are problems attached to this exposition of the concept. Professor Cohen has confounded what philosophers call the context of discovery with the context of justification and has insisted, almost perversely, in using a concept adapted to the context of justification to expound Newton's process of discovery. Few scientists have been less autobiographical in their publications than Newton. He was on the one hand an extremely introverted man who was always concerned to keep his

distance from others. On the other hand, he held advanced standards of rigour and elegance. More than any other scholar, Professor Cohen himself has taught us to appreciate Newton's immense care in his presentation as he continually revised everything he wrote to express his exact meaning. In the present book, he now asks us to receive these same writings as autobiographical accounts of the process by which Newton arrived at his conclusions. Paradoxically, this leads Cohen wholly to reject the validity of genuine autobiographical statements which appear in some of Newton's manuscripts. No doubt the statements were usually self-serving. At best they must be handled with caution, and perhaps some or even all of them need in the end to be dismissed entirely. Surely, however, it is not the published form of non-autobiographical works that can determine what to accept and what to reject.

Cohen's approach leads him to insist that, because Newton expressed reservations, he never seriously entertained the existence of particulate forces in matter and that, because he stated in the *Principia* that gravitational attraction may be performed by impulse, he always believed that the final explanation of gravity would involve a material aether. Cohen's own theory of the Newtonian style applied to the context of justification puts this material in quite a different light. With a remarkably cold eye, Newton distinguished demonstration from speculation, even when the speculation contained some of his most cherished convictions. He chose not to mix mere belief, no matter how deeply held, with what he took to be demonstrations. When we approach his work from this angle, the very concept of a Newtonian style leads us to an account of the development of Newtonian science that is quite different from the one Professor Cohen presents. In my opinion, it is a more convincing account. We see a Newton who was the boldest speculator of the century, a man who was able to break out of the confines of received natural philosophy and to put questions no one else could formulate. The Newtonian style, the separation of speculative explanatory hypotheses from mathematically demonstrated regularities in nature, controlled the speculation by proposing what he could not demonstrate under titles such as "Queries".

Thus I like the notion of a Newtonian style; it gives definite form to important characteristics of Newton that have hitherto not been clearly perceived and articulated. At the same time, I remain to be convinced that the Newtonian style is a useful instrument in the elucidation of Newton's own scientific development. □

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Minerals through the microscope

R.A. Howie

Optical Mineralogy: The Nonopaque Minerals. By Wm Revell Phillips and Dana T. Griffen. Pp.677. ISBN 0-7167-1129-X. (W.H. Freeman: 1981.) £24.60, \$39.95.

ALTHOUGH this book was originally intended to be the companion volume to *Mineral Optics: Principles and Techniques* (1972), to supply the necessary tables of properties and mineral descriptions, it developed into a more comprehensive work. There are two quite distinct parts: Part I contains detailed descriptions of most varieties of the common rock-forming mineral groups, while Part II presents an "abbreviated summary" (pp.399-665) of the optical properties of "all" non-opaque mineral species (including some first described as recently as 1979). The optical descriptions are illustrated with orientation sketches and determinative graphs of optical properties against composition, though unfortunately refractive indices are given as n_o , n_e etc. rather than as α , ϵ as recommended by the International Mineralogical Association. For a small proportion of the minerals there are diagrams of the crystal structure, but although the compositional ranges are discussed there are no phase diagrams at all (even for the Al_2SiO_5 system of the plagioclase series). Notes on zoning, alteration, distinguishing features and occurrence are included, together with a few selected references.

In addition to summaries of the optical and physical constants of the non-opaque minerals, Part II also gives the typical habit, colour (in thin-section and in hand specimen), alteration and occurrence, together with remarks (solubility, group, polymorph etc.) and one reference. The authors state in their preface that it is hoped that this volume will be accepted as the logical successor to A.N. Winchell's

Elements of Optical Mineralogy, II: Descriptions of Minerals (Wiley, 1951) and Larsen and Berman's *The Microscopic Determination of the Nonopaque Minerals* (US Geological Survey, 1934), both long out of date and out of print. Comparison might also be made with Kostov's *Mineralogy* (author's translation edited by P.G. Embrey and J. Phemister), published by Oliver & Boyd in 1968. Certainly, Part II of the present work will prove invaluable. With the rapid discovery of new minerals (currently at the rate of around 80 each year) no single work can ever contain all critical data on all non-opaque minerals, but mineralogists will be in debt to Phillips and Griffen for some years.

In Part I, the detailed descriptions of the commoner rock-forming minerals are well-presented, yet surely are not quite detailed enough. Comparison is inevitably to be made with Battey's *Mineralogy for Students* (Oliver & Boyd, 1972) or Deer, Howie and Zussman's *Introduction to the Rock-Forming Minerals* (Longmans, Green, 1966). Admittedly the present work is concerned with optical mineralogy, but it does include sections on the occurrence of the various minerals and these are not always sufficient for a petrologically inclined mineralogist, e.g. for the garnet group. Nor are the references particularly up-to-date — the most recent augite reference is to a 1970 paper, and for biotite, 1966, though perhaps this is merely a reflection of the increasing use of X-ray and electron microprobe techniques in preference to those of optical mineralogy. The amphibole sections take no account of the IMA rulings (1978) for the nomenclature of this group. Nevertheless this descriptive part of the book is well-produced and attractively presented with numerous clear diagrams and quite a few microphotographed pairs (taken with plain-polarized light and with crossed polars). It must be said, however, that after the colour photographs in MacKenzie and Guilford's recent *Atlas of Rock-forming Minerals in Thin Section* (Longman, 1980), these black-and-white photographs seem uninspiring.

Perhaps the biggest surprise about this book is the juxtaposition of the two parts which would seem to cater for rather different levels of readership. The listing of properties of all known non-opaque minerals in Part II will appeal to final-year honours students and particularly to research workers at all levels, whereas the mineral descriptions in Part I would, in my opinion, be insufficient for a final year honours course in geology. It is, however, remarkably good value at under £20; all libraries catering for the earth sciences should have it. □

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Science and the President. Pergamon Press have recently issued in paperback *Science Advice to the President*, a volume tackling many of the same questions raised by Harvey Brooks in his review of *Science at the White House* by E.J. Burger (*Nature* 290, 634). The book is a collection of 23 essays by all but one of the past Science Advisers and by other well-qualified individuals, edited by William T. Golden. Prices are hbk £25, \$50; pbk £5, \$9.95.

British Anthozoa. In his review of *British Anthozoa* (*Nature* 291, 520) Sir Maurice Yonge regretted the absence of colour illustrations from the book. R.L. Manuel has compiled another guide to anthozoans which contains colour photographs of all but three of the British species. The book was published last year by the Underwater Conservation Society and is available from UCS Sales, 148 College St, Long Eaton, Nottingham NG10 4GX, price £16.

Hennig in English: hypotheses for entomology's systematists

R. A. Crowson

Insect Phylogeny. By W. Hennig. Translated and adapted by A. C. Pont, with revisionary notes by D. Schlee. Pp. 514. ISBN 0-471-27848-3. (Wiley: 1981.) £28, \$84.

WILLI Hennig's 1969 volume, *Die Phylogenie der Insekten* (published by Waldemar Kramer), while undoubtedly a work of scholarly importance, received rather less than due attention in the English-speaking world. At the time of his death in 1976, Hennig was accumulating notes for a revised English-language version of the work. In its lieu, the present volume provides a translation of the 1969 text into good and readable English, interspersed with Hennig's surviving notes for a revision, and supplementary notes by younger, mainly German entomologists designed to bring into consideration work

published since 1968. The bibliography now includes some references as recent as 1980.

The "methodological introduction" summarizes Hennig's phylogenetic methods, more fully set out in his *Phylogenetic Systematics* (University of Illinois Press, 1966). It is followed by a review of Palaeozoic and Mesozoic fossil data on insects, a systematic account of the phylogenetic relations of modern insects down to about order or suborder level, and a more detailed review of the phylogenetic relations of Mesozoic insects. Illustrations are mainly dendrograms or pictures of wings; the bibliography is extensive though far from exhaustive.

The main phylogenetic conclusions, not significantly changed by the later annotations, agree largely with those of Boudreaux's *Arthropod Phylogeny with*

Special Reference to Insecta (Wiley, 1979) who also applied Hennigian methods, though Hennig goes into more detail. A notable feature is the use of a numerical system denoting taxa, based on Hennig's belief that phyletic splittings are almost always binary, these numbers being directly convertible into a topologically correct dendrogram. One effect of the binary system is the use of a large number of classificatory levels — thus it takes eight splittings to get from Insecta (= Hexapoda) to an order such as Diptera, though only two to Diplura.

Perhaps the most important of the additions are due to Dieter Schlee, who brings into consideration a good deal of recently described fossil evidence, not known to Hennig in 1969. Otherwise, the later annotators seem mainly concerned with promulgating their own views about particular orders, not always in a very scholarly manner. Some of these writers, and even Hennig himself, may at times be accused of unduly dogmatic assertion — thus Hennig states that the theory of a blood-sucking ancestor for Diptera "is certainly not correct"; Mickoleit writes "There is no longer any doubt that the Mecoptera are a monophyletic group"; and Kinzelbach states flatly that "Strepsiptera have no evident gula". Hennig himself, and M. Baehr, are both guilty of misunderstanding and misrepresenting my own views on the Coleoptera at some points.

For the non-entomological systematist, the work should be of interest as the example of Hennig's own application of his method to higher-level classification of a major section of the animal kingdom. The data used in this work are drawn almost entirely from the fields of classical comparative anatomy and palaeontology; its conclusions should be taken as scientific hypotheses, which could be tested against evidence from fields such as "macromolecular systematics" not taken into account by Hennig and his co-workers.

It is to be regretted that neither Hennig nor his annotators have tried seriously to establish ancestral modes of life for the taxa with which they are concerned. The result is that the book has little of interest to the new, rising school of palaeoecologists, or even to palaeogeographers, despite Hennig's own interest in geographical distribution as evidence for phylogeny. The main users of the book will undoubtedly be entomologists and palaeoentomologists, for whom this well produced volume should be a desirable acquisition.

Forms of magnetic behaviour

B. R. Coles

Introduction to the Magnetic Properties of Solids. By A.S. Chakravarty. Pp. 696. ISBN 0-471-07737-2. (Wiley: 1981.) £35, \$81.

MAGNETISM continues to present challenges to our physical insight and abilities to produce firmly based formal theories. With its wide range of phenomena and richness in relevant concepts (spin, exchange, spin-waves, domains, ferromagnetism, spin density waves, crystal fields and so on), it is a much more difficult subject to present to the student in an orderly fashion than, on the one hand, classical electricity and magnetism or, on the other, quantum mechanics. Faced with this dilemma authors either accept the complexity and diversity of the real world of the magnetic properties of matter or turn their back on it until they have laid the formal theoretical foundations which they are convinced will ultimately be the basis of explanations of the behaviour of all magnetic matter. As this book (which is firmly in the second category) shows, the difficulty of the latter approach is that the materials and properties described tend less to be those of intrinsic interest than those that illustrate the bits of theory that can be tackled fairly rigorously. It contains very few figures showing the behaviour of real materials (the first is on p.150 and the second on p.265) and those are chosen solely to compare with rather specific calculations rather than because of their power to illustrate important effects.

For the theoretical physicist who seeks

interesting problems that give promise of possible solutions with existing techniques, this will be a helpful introduction that moulds his thinking along the lines that have been thoroughly explored and found useful. It does not, however, give the introduction promised in the title to the magnetic properties of real solids in all their diversity or even to some modern theoretical developments. Some omissions are understandable, given the tenor of the book, but it is strange to find a transition from an account of the band theory of solids to theories of itinerant ferromagnetism — which oddly fails to refer the student to Herring's classic text (*Magnetism* edited by G. Rado and H. Suhl, Vol IV; Academic Press, 1972) — with no reference to the susceptibilities of metals that do not become magnetically ordered. The treatment of spin-wave spectra is surprisingly brief and there is no discussion of the powers of neutron scattering in their investigation. There is also no discussion of the phase transition aspects of magnetism which are currently a focus of much theoretical effort — "critical phenomena" and "renormalization group" do not appear in the index. It is a pity that space that might have introduced even these theoretical aspects was taken up with extended treatments of standard background theoretical formalism which are easily found elsewhere.

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